MEDICAL LIBRARY

JUN 13 1947

Vol. 2. No. 2. JUNE 1947

BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

EDITED FOR

THE BRITISH PHARMACOLOGICAL SOCIETY

BY

J. H. BURN F. HAWKING

N. MUTCH C. M. SCOTT F. R. WINTON

J. H. GADDUM (Chairman) H. R. ING (Secretary)

In co-operation with the EDITOR of the British Medical Journal

LONDON

BRITISH MEDICAL ASSOCIATION

TAVISTOCK SQUARE, W.C.1

YEARLY SUBSCRIPTION (4 NUMBERS) 25s. U.S.A. $6.00 SINGLE NUMBER 7s. 6d.

NOTICE TO CONTRIBUTORS

The JOURNAL, which appears quarterly, publishes original work in all branches of pharmacology and experimental chemotherapy, including the biochemical and pathological aspects of the subject. Provision is also made for short communications and for brief notes on technique. Papers submitted for publication and all editorial communications should be addressed to Dr. H. R. Ing, Department of Pharmacology, Oxford.

Papers submitted to the JOURNAL are accepted on the understanding that they have not been and will not be published in whole or in part in any other journal; they are subject to editorial revision.

MSS. and illustrations should bear the name and full address of the author to whom proofs are to be sent. Papers should be in double-spaced typing on one side only of quarto or foolscap sheets, with a margin of not less than 1½ in. Papers must be as concise as possible, and only recognized abbreviations should be used.

In stating the object of the research the author should refer to previous work only if it has a direct bearing on the subject of the paper; as a rule a complete historical review is unnecessary. A paper should conclude with a brief but self-explanatory summary giving in numbered paragraphs the results and conclusions described in the paper; it should not exceed 1-20th of the length of the paper.

TABLES.—Tables are to be typed on separate numbered sheets and referred to in the text by Roman numerals. Tables should be comprehensible without reference to the text.

ILLUSTRATIONS.—Illustrations are to be referred to in the text by Arabic numerals. Legends or captions are to be typed on separate sheets and numbered correspondingly; they should render the illustrations comprehensible without reference to the text. Line drawings and graphs, which should be about twice their intended final size, are to be carefully drawn in Indian ink on tracing linen, or Bristol board, or stout smooth white paper; blue-lined graph paper of good quality may be used for graphs. Lettering of drawings and graphs must be lightly written in pencil. Lettering on Kymograph records should be large enough to be legible on photographic reproduction. When half-tone reproduction of *x*-ray or other illustrations is required the author is advised to send the original film unless he wishes to bring out special points in a print of his own choice. Photographs and photomicrographs should be printed on glossy paper, should be larger than the size desired for reproduction, and, if transmitted through the post in a tube, should be rolled with the picture outside.

REFERENCES.—References should be arranged according to the Harvard system. In the text the year of publication must follow the author's name, more than one paper in any one year being indicated by a small letter (a,b,c) after the date. At the end of the paper references should be arranged in the alphabetical order of the authors' names and should include: author's name, followed by initials; year of publication, in parentheses; title of publication, underlined, and abbreviated in accordance with the *World List of Scientific Periodicals*; volume number in Arabic numerals underlined with a wavy line to indicate bold type; the number of the first page in Arabic numerals, thus: Barger, G., and Dale, H. H. (1910–11). *J. Physiol.*, 41, 19.

The BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY should be referred to in the list of references as *Brit. J. Pharmacol.* When reference is made to a book the author's name and the date should be followed by: title, underlined; edition, volume number (vol.), and page (p. or pp.); town of origin: and the publisher, thus: Clark, A. J. (1940). *Applied Pharmacology*, 7th ed., p. 378. London; Churchill.

For further information on the preparation of MSS. for publication authors are recommended to study the "Suggestions to Authors" published by the Editorial Board of the *Journal of Physiology*, (1945), **104**, 221.

PROOFS.—Contributors will receive one proof in page, but it will be assumed that all but verbal corrections have been made in the original MS.; an allowance at the rate of ten shillings per sheet of sixteen pages is made for alterations in the proof (printer's errors excepted), and contributors will be charged for any excess.

REPRINTS.—Fifty reprints of an article will, if desired, be given to an author or divided among the authors. A limited number of additional reprints at cost price can be supplied if application is made when the proofs are returned, and an estimate of costs can be obtained from the Publishing Manager, British Medical Association.

COPYRIGHT.—Papers which have been published become the property of the JOURNAL and permission to republish must be obtained from the Editors.

Brit. J. Pharmacol. (1947), **2**, 65.

THE ABSORPTION OF SOME SULPHAGUANIDINE DERIVATIVES IN MICE

BY

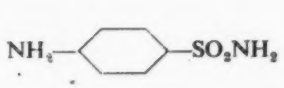
F. L. ROSE AND A. SPINKS

From the Research Laboratories, Imperial Chemical Industries, Ltd., Blackley, Manchester 9

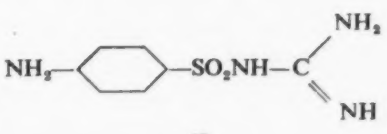
(Received October 17, 1946)

Sulphaguanidine (II) was independently prepared by Marshall, Bratton, White, and Litchfield and Roblin, Williams, Winnek, and English in 1940, and introduced for the treatment of bacillary dysentery on the basis of its poor absorption from the gut. It is now well established that orally administered sulphaguanidine attains only low concentrations in the blood of man (Marshall, Bratton, Edwards, and Walker, 1941 ; Anderson and Cruickshank, 1941 ; Beling and Abel, 1941 ; Frisk, 1941) and animals (Marshall, Bratton, White, and Litchfield, 1940 ; Roblin, Williams, Winnek, and English, 1940 ; Cameron and McOnie, 1941 ; Zozaya, 1941 ; Ambrose and Haag, 1942 ; Rose and Spinks, 1946), and that this is due partly to the absorption of only some 30–50 per cent of the administered drug and partly to a rather rapid rate of clearance by the kidney (Frisk, 1941 ; Zozaya, 1941 ; cf. Rose and Spinks, 1946 ; Fisher, Troast, Waterhouse, and Shannon, 1943). Little light has been thrown on the physical or chemical characteristics responsible for this poor absorption, which might be due either to a slow rate of diffusion across the intestinal membranes or to a partial availability of drug within the gut. Sulphaguanidine is relatively highly soluble compared with, for example, sulphapyridine and sulphathiazole, so that any such partial availability cannot be ascribed to low solubility over the intestinal pH range.

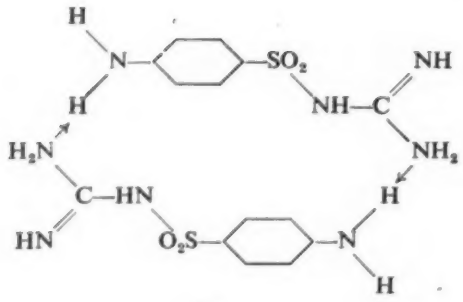
Whatever may be the cause of this phenomenon, it is not immediately apparent how such a striking effect could arise from the simple difference in chemical structure between the parent sulphanilamide, which is very well absorbed, and sulphaguanidine.



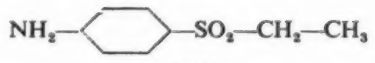
I



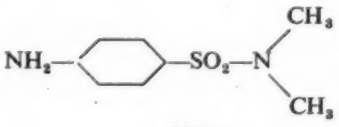
II



IIa



III



IIIa

Sulphanilamide (I) is derived from ammonia by replacement of a hydrogen atom of the latter by the sulphanilyl radicle. Although ammonia is a base, that is, in aqueous solution it acquires a proton to form the ammonium ion, the influence of the sulphonyl group is such that in sulphanilamide this tendency is reversed and the sulphonamide group exhibits feeble acidic properties, that is, it tends to liberate a proton. Guanidine is, however, a much stronger base than ammonia, and in combination with the sulphonyl group provides the sulphonylguanidine group, which is approximately electrochemically neutral ; that is, it is neither markedly acidic nor basic. Krebs and Speakman (1946) have suggested as a corollary to their work on solubility and dissociation constants in the sulphanilamide series that poor absorption from the intestine is a result of this suppression of ionization. In our opinion, it is unlikely that this effect, which constitutes the main chemical difference between sulphanilamide and sulphaguanidine, can be the primary cause of their different pharmacological behaviour, otherwise compounds such as *p*-aminophenylethylsulphone (III) and sulphanildimethylamide (IIIa) which carry the non-ionizing sulphonylethane and sulphonyldimethylamide groups, respectively, might also be expected to resemble sulphaguanidine. As is shown below in Tables I and II, both of these compounds are relatively well absorbed, the former to the same extent as sulphanilamide.

We were therefore led to consider the possibility that a physical difference between sulphaguanidine and sulphanilamide other than that suggested by Krebs and Speakman might account for their observed physiological behaviour.

The work of Hunter (1941) has drawn attention to the capacity of amidine and guanidine groups to take part in hydrogen bond formation, and we tentatively supposed that similar effects involving both the sulphonylguanidine group and the primary amino group in the *para* position of the benzene ring might account for the poor absorption of sulphaguanidine through union with some substrate or substrates to be found in the gut contents. A further factor might be the formation of dimeric molecules of type (IIa) in which, it is suggested, hydrogen bonds again play a part. It is presupposed that in either event, the sulphonylguanidine and *p*-amino groups operate simultaneously, the former as a hydrogen

acceptor and the latter as a hydrogen donor. Any charge displacement resulting from the acceptance of a hydrogen atom in one part of the molecule would then be balanced by an opposite and approximately equal displacement in another.

Union with a substrate would necessarily reduce the amount of drug available for absorption, while the increased molecular weight resulting from dimer formation would probably lead to slower absorption. In the latter connection, sulphanilamide derivatives of molecular weight comparable to that of the hypothetical dimer, which have been studied in these laboratories, for example some sulphanilamidoquinolines, have attained only low concentrations in the blood of mice, and it must be emphasized that what is to be explained is not a complete failure of absorption, but partial absorption to a degree not much less, in some species, than that shown by, say, sulphapyridine.

The first hypothesis, that of union with a substrate, cannot easily be tested by direct experimentation. The second should be capable of proof or disproof by molecular weight determinations in aqueous solution. So far, however, it has not been possible to obtain unequivocal results by such determinations. Indirect evidence for an effect involving two point hydrogen bonding has been sought by examining the absorption in mice of a number of sulphaguanidine derivatives. In these the molecule has been modified so that the possibility of association through hydrogen bonding is either left unchanged or is reduced or wholly eliminated. The several chemical types were selected on the following basis:

Type I. Compounds carrying substituents in the guanidine residue

A twofold influence was anticipated in this instance. The introduction of one or more alkyl groups on the terminal nitrogen atom would enhance the electronegativity of the latter and might therefore be expected to strengthen hydrogen bond formation. Against that, the steric effect of the substituents, depending upon their sizes, should inhibit the necessary close approach to a second molecule, and would probably be the more important factor.

Type II. Compounds in which double hydrogen-bonding is not possible

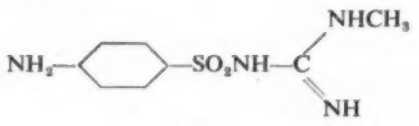
The substitution of nitro for the primary amino group (XI) or the transference of the latter to the *meta* position (XII) would effectively modify the formation of the double hydrogen-bond systems ; in the first compound because the nitro group is devoid of the necessary hydrogen atom, and in the second because of the markedly different relative positions of the significant groups in the drug molecule, rendering impossible, for example, the achievement of a structure such as (IIa). A further compound (XIII) was also included under this heading, in which it was anticipated that the presence of a methyl in the *ortho*

position to the primary amino group might inhibit to some extent the participation of the latter in hydrogen bond formation. The correspondingly substituted sulphanilamide (XIIIa) was examined as a control substance.

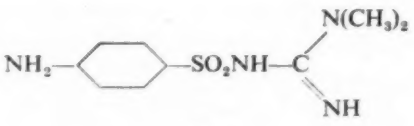
In addition to compounds classified under the above two headings, we included in this investigation several miscellaneous substances such as the two acetyl derivatives of sulphaguanidine (XIV) and (XV), and the sulphonyl acetamidine (XVI) which closely resembled sulphaguanidine chemically, and which could be involved in hydrogen bond formation in the same manner as the latter drug.

The substances employed in this investigation are listed below. For the preparation of some of them we are indebted to our colleagues, Dr. F. H. Slinger and Dr. G. Swain. Details of the chemical work will be described in the *Journal of the Chemical Society*.

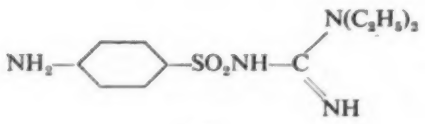
Type I



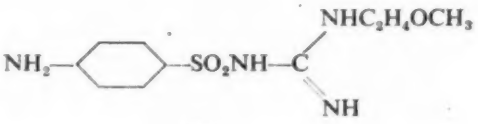
IV



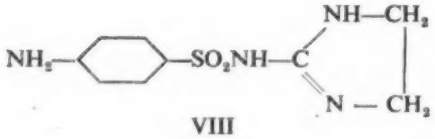
V



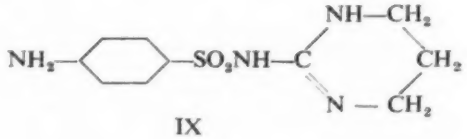
VI



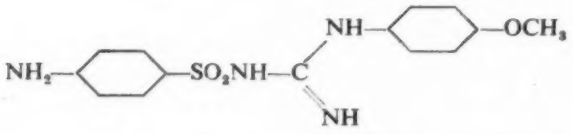
VII



VIII

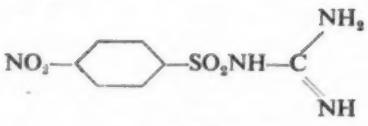


IX

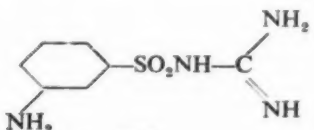


X

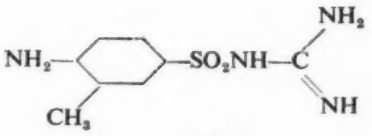
Type II



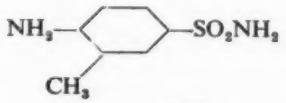
XI



XII

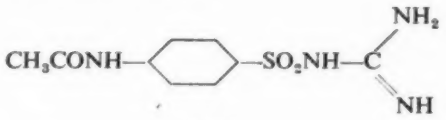


XIII

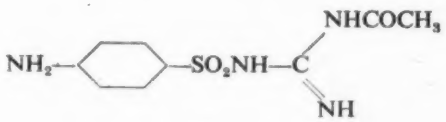


XIIIa

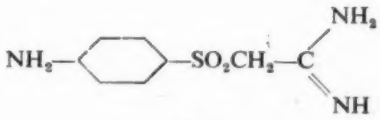
Miscellaneous



XIV



XV



XVI

A few compounds of Type I have been described by Winnek, Anderson, Marson, Faith, and Roblin (1942), who also recorded the maximum blood concentrations attained following the oral administration to mice of single doses of 0.5 g./kg. They concluded from experiments with the ethyl, propyl, and butyl homologues of (IV) that the presence of the alkyl groups led to an increased absorption, the increase being particularly marked in the case of the propyl derivative.

EXPERIMENTAL

1. Analytical methods

With two exceptions (XI and XIV) each compound was determined by the micro method of Rose and Bevan (1944). The advantages of this method have recently been summarized elsewhere (Spinks and Tottey, 1946). Adequate recoveries (± 10 per cent) of each compound were established by trial analyses of known amounts added to blood.

p-Nitrobenzenesulphonylguanidine was determined by the following method based on that of Eckert (1943) for *p*-nitrobenzoic acid. 0.02–0.04 ml. blood was measured and pipetted into 0.4 ml. water in a test tube (18×100 mm.), and 0.2 ml. trichloroacetic acid added. After centrifugation, the clear supernatant fluid was decanted into a similar tube graduated at 2 ml., the protein residue being washed with 0.2 ml. water: one drop of Eckert's tartaric acid mixture was then added, followed by 1 drop of *N*/20 titanous sulphate, and the tube was heated for 10 min. at 100° C. On cooling, 0.2 ml. 0.10 per cent sodium nitrate was added, and, 20 min. later, 0.4 ml. of 1 per cent N-β-sulphatoethyl-*m*-toluidine (Rose and Bevan, 1944). Thirty min. were allowed for coupling and the volume was then made up to the mark with distilled water. Immediately before a reading in the colorimeter each tube was centrifuged, since some turbidity was usually evident. This procedure was found to give only 70–80 per cent recoveries of *p*-nitrobenzenesulphonylguanidine from

blood. Eckert obtained similar results with *p*-nitrobenzoic acid. Instead of using a conversion factor, unknown samples were read against standards (0, 0.01, 0.03, 0.05, 0.07, and 0.10 ml. volumes of a 10 mg./100 ml. aqueous solution of *p*-nitrobenzenesulphonylguanidine), to which blood (0.04 ml.) had been added. In spite of the centrifugation immediately before colorimetry individual blood concentration-time curves obtained for *p*-nitrobenzene-

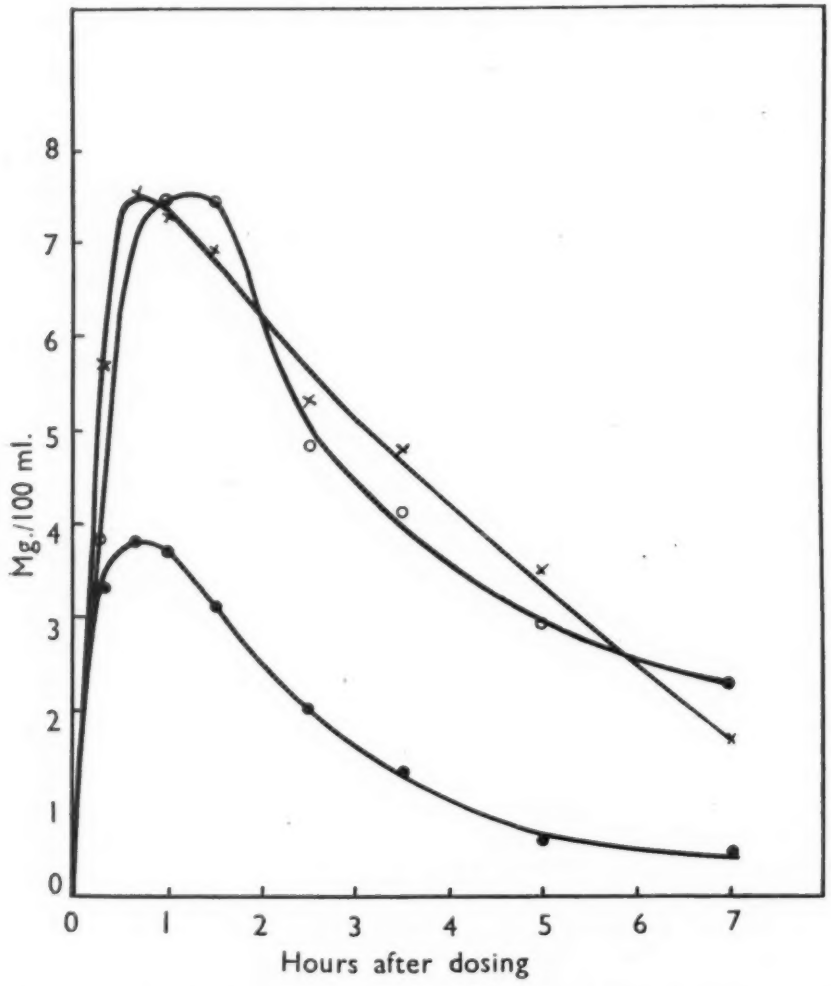


FIG. 1.—Mean blood concentration-time curves in mice of sulphaguanidine (II, ●——●, 30 animals), metanilylguanidine (XII, O——O, 18 animals), and *p*-nitrobenzenesulphonylguanidine (XI, ×——×, 24 animals). Each compound was administered by stomach tube in doses of 5 mg./20 g.

sulphonylguanidine were not very smooth, and it is thought that, owing to residual turbidity, the error of the method may have been as high as 20 per cent. However, the mean curve (Fig. 1) was adequately smooth. The final result, of course, included the *p*-nitrobenzenesulphonylguanidine that had been converted to sulphaguanidine *in vivo*. The extent of such

conversion was separately examined, the blood from mice receiving 5 mg. of *p*-nitrobenzenesulphonylguanidine per 20 g. being analysed for sulphaguanidine itself. No sample contained more than 0.5 mg./100 ml. of the latter. *p*-Acetamidobenzenesulphonylguanidine (XIV) was determined by the method of Rose and Bevan (1944) for total amine, modified so that the protein precipitation was carried out at about 1 in 50 dilution instead of 1 in 20. This involved an increase in the final volume of solution to 5 ml., but satisfactory sensitivity was achieved by reading in 2 cm., 6 ml. cells in the Morris colorimeter.

II. Biological methods

The standard conditions used by us in the examination of sulphonamide blood concentrations in mice have already been described (1946), and the original publication should be consulted for full details. Each compound was administered orally to a group of 3 mice in doses of 5 mg./20 g. and as a 1 per cent solution (of the hydrochloride) or dispersion, pooled tail blood from the 3 animals being analysed at intervals after dosing.

This individual experiment was repeated at least 6 times for each drug, and characteristic values obtained from the individual smooth curves as already described (Rose and Spinks, 1946), including maximum concentration (max.), the time after dosing at which this was attained (t.max.), and the time (C.5) taken for the concentration at 5 hours to fall to two-thirds of the value then observed. The results are given in Table I. The mean results are summarized in Table II, in which the compounds have been arranged in order of falling maximum blood concentration. Limits of error of means are for a probability level of 0.05 ; that is, there is one chance in twenty of the error exeeding the given limit. A value differing significantly ($P=0.05$) from that for sulphaguanidine is printed in italics, one differing decisively ($P=0.01$) in bold type.

Two compounds only (IV and X) differed significantly from sulphaguanidine in rate of disappearance from the blood. It may therefore be concluded that the maximum blood concentration is a fairly satisfactory index of the extent of absorption from the gut.

TABLE I

BLOOD CONCENTRATIONS OF SULPHAGUANIDINE DERIVATIVES AND ALLIED COMPOUNDS

Compound No.	No. of experiments	Mean blood concentrations in mg./100 ml. after									Values from individual curves		
		20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.	Max. (mg./100 ml.)	t max. (min.)	C.5 (hr.)
III	7	7.7	10.3	10.6	10.6	7.2	3.8	1.7	0.9	0.1	11.7	63	1.1
	Standard deviations										3.0	21	0.5
	Limits of error of means										±2.7	±19	±0.5
IIIa	8	6.1	8.0	8.3	7.8	6.5	4.2	3.15	1.2	0	8.9	71	1.2
	Standard deviations										1.6	19	0.6
	Limits of error of means										±1.3	±15	±0.45

TABLE I—*continued.*

Compound No.	No. of experiments	Mean blood concentrations in mg./100 ml. after:—									Values from individual curves		
		20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.	Max. (mg./100 ml.)	t max. (min.)	C.5 (hr.)
IV	9	2.8	3.8	3.9	3.8	2.7	1.8	1.1	0.8	0.2	3.9	67	1.3
	Standard deviations										1.6	8	0.4
	Limits of error of means										±1.2	±6	±0.3
V	6	7.8	8.0	7.3	5.5	3.4	2.25	1.4	1.0	0.2	8.35	42	1.4
	Standard deviations										2.3	24	1.1
	Limits of error of means										±2.3	±24	±1.1
VI	6	5.9	7.05	7.4	7.5	6.3	4.65	3.4	1.9	1.0	7.9	98	2.6
	Standard deviations										1.5	42	1.7
	Limits of error of means										±1.5	±42	±2.0
VII	8	3.2	6.0	5.5	4.6	3.2	2.1	1.2	0.95	0.3	5.7	72	1.6
	Standard deviations										1.45	13	1.0
	Limits of error of means										±1.2	±11	±0.9
VIII	7	4.3	6.0	7.3	7.1	5.6	3.6	2.3	1.2	0.2	7.3	85	1.3
	Standard deviations										2.0	39	0.4
	Limits of error of means										±1.8	±36	±0.4
IX	6	3.35	5.3	5.3	5.2	4.3	2.4	1.2	0.8	0.3	5.6	74	1.3
	Standard deviations										1.3	23	0.6
	Limits of error of means										±1.3	±23	±0.6
X	6	4.6	4.7	3.7	3.2	1.35	0.6	0.15	0.1	0	5.1	37	0.6
	Standard deviations										1.6	4.5	0.2
	Limits of error of means										±1.8	±5.2	±0.2

TABLE I—*continued.*

Compound No.	No. of experiments	Mean blood concentrations in mg./100 ml. after:—									Values from individual curves		
		20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.	Max. (mg./100 ml.)	t max. (min.)	C.5 (hr.)
XI	8	5.7	7.5	7.3	6.9	5.3	4.8	3.5	1.7	0	7.6	53	1.7
	Standard deviations										1.8	18	1.6
	Limits of error of means										±1.5	±15	±1.3
XII	6	3.8	7.1	7.7	7.7	4.8	4.1	2.9	2.25	0.6	7.8	82	2.0
	Standard deviations										3.2	35	1.2
	Limits of error of means										±3.2	±35	±1.2
XIII	6	2.55	2.9	2.75	2.4	1.6	1.6	0.85	0.5	0.2	3.1	48	1.3
	Standard deviations										0.8	18	0.4
	Limits of error of means										±0.8	±18	±0.4
XIIIa	6	10.5	13.1	13.3	12.9	10.6	6.8	5.0	2.9	0.1	13.5	63	1.4
	Standard deviations										3.8	14	0.4
	Limits of error of means										±3.8	±14	±0.4
XIV	6	1.1	1.3	1.4	1.4	1.7	1.55	1.2	0.7	0.4	1.95	127	(3.2*)
	Standard deviations										0.7	51	—
	Limits of error of means										±0.7	±59	—
XV	6	3.6	4.8	4.7	4.2	3.7	2.5	2.0	1.0	0	4.75	67	1.3
	Standard deviations										2.0	16	0.7
	Limits of error of means										±2.0	±16	±0.7
XVI	9	1.4	2.1	2.3	2.0	1.6	1.2	0.8	0.5	0.1	2.7	75	1.3
	Standard deviations										1.1	34	0.95
	Limits of error of means										±0.85	±25	±0.7

*Does not differ significantly from 0; i.e., no mean is justifiable.

TABLE II

SUMMARY OF MEAN DATA ON THE ABSORPTION OF SULPHAGUANIDINE DERIVATIVES AND ALLIED COMPOUNDS

Figures differing significantly (P = 0·05) from those for sulphaguanidine in italics, figures differing decisively (P = 0·01) in bold type.

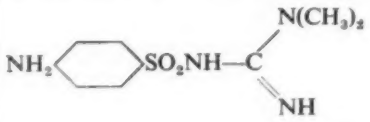
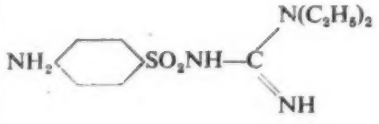
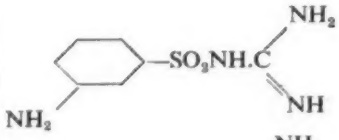
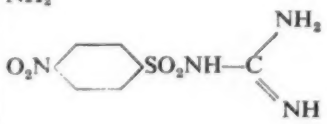
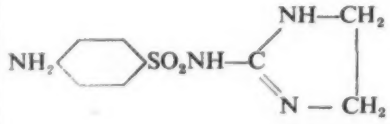
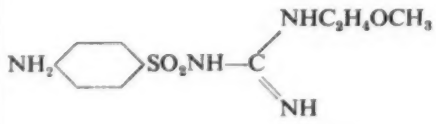
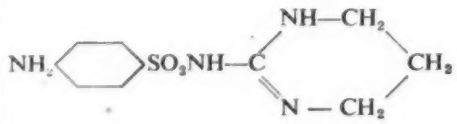
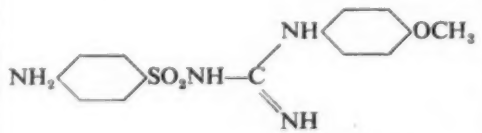
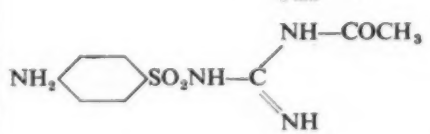
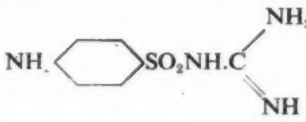
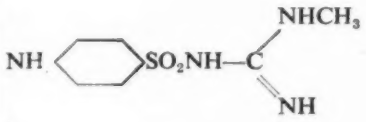
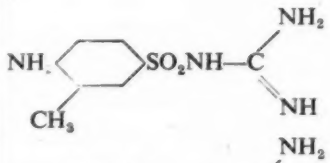
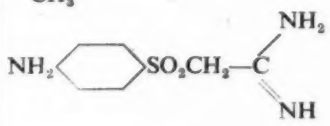
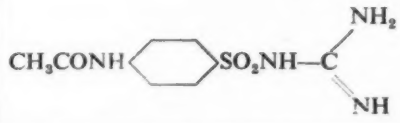
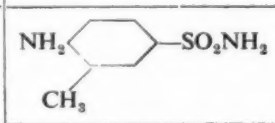
No.	Formula	Max. (mg./100 ml.)	t max. (minutes)	C.5 (hours)
V		**8·35 ± 2·3**	42 ± 24	1·4 ± 1·1
VI		**7·9 ± 1·5**	**98 ± 42**	2·6 ± 2·0
XII		**7·8 ± 3·2**	82 ± 35	2·0 ± 1·2
XI		**7·6 ± 1·5**	53 ± 15	1·7 ± 1·3
VIII		**7·3 ± 1·8**	85 ± 36	1·3 ± 0·4
VII		*5·7 ± 1·2*	**72 ± 11**	1·6 ± 0·9
IX		*5·6 ± 1·3*	*74 ± 23*	1·3 ± 0·6
X		5·1 ± 1·8	37 ± 5·2	**0·6 ± 0·2**
XV		4·75 ± 2·0	67 ± 16	1·3 ± 0·7

TABLE II—*continued.*

No.	Formula	Max. (mg./100 ml.)	t max. (minutes)	C.5 (hours)
II		4·3 ± 0·8	50 ± 12	2·2 ± 0·85
IV		3·9 ± 1·2	*67 ± 6*	*1·3 ± 0·3*
XIII		3·1 ± 0·76	48 ± 18	1·3 ± 0·4
XVI		**2·7 ± 0·85**	*75 ± 25*	1·3 ± 0·6
XVI		*1·95 ± 0·7*	**127 ± 59**	—
XIIIa*		13·5 ± 3·8	63 ± 14	1·4 ± 0·4
III*		11·7 ± 2·7	63 ± 19	1·1 ± 0·5
IIIa†		**8·9 ± 1·3**	*71 ± 15*	1·2 ± 0·45

* Neither III nor XIIIa differs significantly from sulphanilamide in any respect.

† The significant differences indicated are from sulphaguanidine. The maximum blood concentration is decisively lower than that of sulphanilamide. There is no other significant difference from sulphanilamide.

Discussion of Results

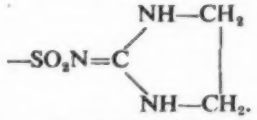
The hypothesis on which this research was based is that sulphaguanidine and related compounds exhibiting poor absorption from the gut owe this property to those features of their molecular structure which permit union with some hypothetical substrate, with the additional possibility of self union to provide a dimeric molecule. It is further supposed that in either event the linking forces are provided by hydrogen bonds associated with the *p*-amino and guanidine groups. The *para* configuration of the sulphaguanidine molecule would be of

special significance in dimer formation, and it is assumed that it might also be of importance in unions of the type postulated with other molecules. These structural features should be reflected in the physical properties of the drug molecules and be capable of measurement. This aspect is receiving the attention of our colleague, Mr. J. C. Gage.

It is convenient to discuss the results obtained under the type headings listed above, noting in each case the extent to which the disturbance of molecular detail has produced the expected effect.

Type I

Of the alkyl derivatives of sulphaguanidine only the monomethyl homologue gives a maximum blood concentration lower than that of the parent compound, and the difference is not significant. The remaining alkyl derivatives all achieve higher maxima, in particular the dimethyl and diethyl homologues. The contrast between the former and the monomethyl derivative is marked, and, in so far as hydrogen bond formation is concerned, would indicate that the steric rather than the inductive effect of the alkyl groups is the governing factor, the greater hindrance to the necessary close approach to a substrate or a second molecule being provided, as would be anticipated, by the presence of two alkyl groups. The two ring alkylene compounds (VIII) and (IX) provide a further contrast, the former being absorbed the more completely, although somewhat more slowly. Compound (IX), incidentally, is the tetrahydropyrimidine corresponding to sulphadiazine, a drug which under similar conditions achieves an average maximum blood concentration of 17.5 mg./100 ml. (Rose and Spinks, 1946). A theoretical explanation of the difference between (VIII) and (IX) can at this stage be no more than speculation. It is unlikely that the small increase in molecular weight corresponding to the additional methylene group in (IX) is the responsible factor. The main chemical feature of the latter substance would be a tendency for the double bond of the guanidine residue to be stabilized in the heterocyclic ring as formulated, while in (VIII) the disposition of the double bond giving the minimum strain in the ring system might be expected to be that provided in



These differences would almost certainly influence the relative hydrogen-bonding propensities of the ring nitrogen atoms, and be reflected in different degrees of absorption.

Type II

The *meta* isomer of sulphaguanidine (XII) attains a maximum blood concentration which is approximately double that of the parent compound. Some slight change in physical properties (base dissociation constant, solubility, etc.)

would be expected in passing from the one isomer to the other, but it is unlikely that these differences would be solely responsible for the marked increase in absorption of the *meta* compound. At the same time the maximum concentration attained is below the known value for sulphanilamide (13.2 mg./100 ml.). It would appear, therefore, that the sulphonylguanidine grouping is *per se* less favourable to absorption from the gut than the sulphonamide group and that this inhibiting effect is markedly increased in association with a *para* amino group. The close identity of the absorption data of (XII) and the nitro compound (XI) (see Fig. 1) adds further support to these views. In the latter substance a *para* substituent is present, but it is of a chemical type unable to provide hydrogen atoms for bond formation.

The presence of a methyl group *ortho* to the amino group of sulphaguanidine (XIII) suppresses rather than enhances absorption. Reference to formula (IIa) shows that for the methyl group to inhibit hydrogen bonding to any marked extent, the molecules must be oriented with the alkyl groups adjacent to, and on the same side as, each of the two hydrogen bonds. No inhibition would be expected with the methyl groups in the alternative *ortho* positions. The actual experimental results are inconclusive, therefore, as regards substantiation of the hypothesis. It should be noted that compound (XIIIa), which was included as a control substance in this aspect of the investigation, does not differ significantly in its absorption characteristics from sulphanilamide (Rose and Spinks, 1946).

Among the miscellaneous substances included in this research, the results obtained with the sulphonylacetamidine (XVI) are of some significance. This compound differs structurally from sulphaguanidine only in that the imido group of the latter is replaced by a methylene linkage. The terminal amidine residue $-C(=NH)-NH_2$ is common to both. Since both compounds are poorly absorbed, it would appear that the amidine moiety is the controlling feature. This is in accord with expectations. On the other hand, the experimental findings with the acetyl derivative of sulphaguanidine (XV) were not anticipated. The combined influence of the sulphonyl and acetyl groups on the guanidine residue is such that this substance regains the weak acidic properties characteristic of the simple sulphonamide group. The guanidine group in (XV) would, therefore, be expected to function less readily as a hydrogen acceptor and the compound should then resemble sulphanilamide rather than sulphaguanidine. Against this, it has been observed that the acetyl group of (XV) is removed with unusual ease, for example, on standing for a short period of time in cold dilute sodium hydroxide solution, to regenerate sulphaguanidine, and it may be that such deacetylation occurs in the gastro-intestinal tract resulting in an observed absorption characteristic of the latter drug.

In conclusion, it is apparent that the results of the experiments *in vivo* recorded above can at best provide only indirect substantiation of the hypothesis proposed. Further, with compounds such as the alkylguanidine derivatives, two

effects were expected to result from the introduction of the alkyl groups, the one tending to decrease, the other to increase the stability of hydrogen bond formation, but in the absence of the necessary physical data relating to these substances it was not possible to forecast with certainty which feature would predominate. However, the marked contrast between the absorption of sulphaguanidine and, for example, its diethylhomologue (VI), in which the molecular weight is increased by nearly sixty units, would be contrary to expectations were it not postulated that the action of the alkyl groups is one of steric hindrance to the approach to the guanidine residue of some other chemical structure which by its presence would inhibit passage of the drug through the gut wall. The increased absorption noted by Winnek *et al.* (1942), in particular of the monopropyl derivative of sulphaguanidine, can also be accounted for on the same basis. The conclusions to be drawn from the data for the *p*-nitro (XI) and *m*-amino (XII) compounds are more precise. Clearly the capacity of these compounds to be involved in association with a second structure in which the potential hydrogen bonding is oriented to accommodate the *p*-aminobenzenesulphonyl guanidine molecule will be markedly influenced in these two instances in favour of more ready absorption from the gut.

Summary

The blood concentrations, following oral administration to mice, of a number of derivatives of sulphaguanidine have been determined, and the results analysed in the light of the hypothesis that the poor absorption from the gut of the parent compound is associated with structural features which may permit this substance to exhibit hydrogen bond phenomena. While much of the experimental evidence circumstantially supports this view, some of the results are inconclusive.

References

Ambrose, A. M., and Haag, H. B. (1942). *Surgery*, **12**, 919.
Anderson, O. E. W., and Cruickshank, R. (1941). *Brit. med. J.*, **2**, 497.
Beling, C. A., and Abel, A. R. (1941). *J. med. Sci., New Jersey*, **38**, 629.
Cameron, H. S., and McOnie, W. A. (1941). *Cornell Vet.*, **31**, 321.
Eckert, H. W. (1943). *J. biol. Chem.*, **148**, 197.
Fisher, S. H., Troast, L., Waterhouse, A., and Shannon, J. A. (1943). *J. Pharmacol.*, **79**, 373.
Frisk, A. R. (1941). *Acta med. scand.*, **109**, 355.
Hunter, L. (1941). *J. chem. Soc.*, 777.
Krebs, H. A., and Speakman, J. C. (1946). *Brit. med. J.*, **1**, 47.
Marshall, E. K., Bratton, C., Edwards, L. B., and Walker, E. (1941). *Johns Hopk. Hosp. Bull.*, **68**, 94.
Marshall, E. K., Bratton, C., White, H. J., and Litchfield, J. T. (1940). *Johns Hopk. Hosp. Bull.*, **67**, 163.
Roblin, R. O., Williams, J. H., Winnek, P. S., and English, J. P. (1940). *J. Amer. chem. Soc.*, **62**, 2002.
Rose, F. L., and Bevan, H. G. L. (1944). *Biochem. J.*, **38**, 116.
Rose, F. L., and Spinks, A. (1946). *J. Pharmacol.*, **86**, 264.
Spinks, A., and Tottey, M. M. (1946). *Ann. trop. Med. Parasit.*, **40**, 101.
Winnek, P. S., Anderson, G. W., Marson, H. W., Faith, H. E., and Roblin, R. O. (1942). *J. Amer. chem. Soc.*, **64**, 1682.
Zozaya, J. (1941). *Ciencia (Mex.)*, **2**, 255.

Brit. J. Pharmacol. (1947), **2**, 79.

THE EFFECTS OF PYRIMIDINE SULPHONAMIDE DERIVATIVES UPON THE BLOOD-CLOTTING SYSTEM AND TESTES OF CHICKS AND THE BREEDING CAPACITY OF ADULT FOWLS

BY

F. D. ASPLIN AND E. BOYLAND

From the Veterinary Laboratory, Ministry of Agriculture and Fisheries, Weybridge, and the Chester Beatty Research Institute, The Royal Cancer Hospital (Free), London, S.W.3

(Received November 19, 1946)

Caecal coccidiosis in chickens can be effectively treated with certain sulphonamides, particularly sulphamezathine (sulphadimethylpyrimidine) (Horton-Smith and Taylor, 1943) and sulphapyrazine (Horton-Smith and Boyland, 1946). Sulphamezathine was extensively used in the treatment of outbreaks of caecal coccidiosis on poultry farms during the 1945 and 1946 seasons. On some of these farms a few chicks did not thrive during treatment and a small proportion died. The only apparent abnormality found on post-mortem examination of such chicks was the presence of haemorrhages of the intestines and occasionally of the liver and skeletal muscles. The appearance of such chicks resembled that described in vitamin K deficiency and the haemorrhagic form of sweet clover disease. In order to investigate this syndrome, sulphamezathine was dosed to chicks experimentally and was found to produce hypoprothrombinaemia in most chicks. Internal haemorrhages, similar to those found in the field, were observed in a small proportion of cases.

In the course of these experiments cockerels dosed with sulphamezathine for periods longer than 10 days showed premature development of combs and wattles ; such cockerels had enlarged testes with hypertrophy of the seminiferous tubules. A brief account of these effects has been published (Asplin, Boyland, and Horton-Smith, 1946).

Experimental

Except where otherwise stated Rhode Island Red (R.I.R.) chicks of mixed sexes or Light Sussex × Rhode Island Red cockerels were kept on dry standard mash or placed on experimental diets when one week old. The sulphonamides were administered as solutions of the sodium salts in the drinking water. In some experiments chicks were weighed at weekly intervals to determine the effect of treatment on body growth. Blood from recently killed chicks was taken by heart puncture and placed in tubes containing sodium oxalate. The plasma was separated by centrifugation and the prothrombin time determined by a modification of the method of Witts and Hobson (1940) ; the only change in the method was the omission of lecithin. The prothrombin times were determined on undiluted plasma

and on plasma diluted four times with saline, in the presence of Russell viper venom (0.004 per cent (w/v)) and $CaCl_2$ (0.1 M.).

A lengthening of clotting time was observed regularly in chickens dosed with 0.2 per cent (w/v) sulphamezathine; in some cockerels, similarly dosed, there was also enlargement of the combs and wattles as shown in Fig. 1. Cockerels with enlarged combs invariably had enlarged testes, which was clearly shown when the individual gonad weights were expressed in mg. per 100 g. body weight.

TABLE I

THE EFFECT OF ADDITION OF VARIOUS SULPHONAMIDES TO THE DRINKING WATER OF GROUPS EACH OF 7 YOUNG CHICKS FOR 28 DAYS

All values are means for the groups

Drug 0·2 per cent (w/v)	Sex	Body weights at end of dosing g.	Haemoglobin g. per 100 ml.	Prothrombin time (sec.)		Gonad weight (mg.)	
				undil.	dil. × 4	actual	mg. per 100 g. b. wt.
Sulphadiazine	M	255	8·9	26·4	47·3	29·7	11·6
	F	282				136·5	48·0
Sulphathiazole	M	321	10·2	26·2	47·2	48·3	15·0
	F	284				133·7	47·1
Sulphamerazine	M	290·2	9·5	27·3	49·6	54·6	18·8
	F	228·8				122·3	53·4
Sulphamezathine	M	224·5	8·9	36·2	81·4	63·1	28·1
	F	265·5				149·5	56·3
Sulphapyrazine	M	293·4	9·3	22·9	43·0	30·9	10·5
	F	315·8				146·8	43·3
Water control	M	275·4	9·8	23·2	41·8	39·4	14·3
	F	289·2				137·2	47·5

Results of a typical experiment in which groups, each of seven 7-day-old R.I.R. chicks, were dosed with different sulphonamides are shown in Table I. Sulphamezathine treatment appears to reduce body growth more than any of the other drugs and has a much greater effect on prothrombin clotting time and testes weight than any of the other compounds tested. None of the compounds used

TABLE II

EFFECT OF ADMINISTRATION OF SULPHAMEZATHINE AND *p*-AMINOBENZOIC ACID IN DRINKING WATER FOR 28 DAYS ON TESTES WEIGHT AND PLASMA PROTHROMBIN OF COCKERELS

Treatment	No. of chicks	Prothrombin time sec. (means) undil. plasma	Testes weight (mg.) (means)	
			actual	mg. per 100 g. body wt.
Sulphamezathine 0·2%	6	43·3	51·6	24·2
Sulphamezathine 0·2% + *p*-aminobenzoic acid 0·02%	6	39·3	77·4	34·4
p-aminobenzoic acid 0·02%	6	26·3	43·8	13·7
Water control	6	26·1	34·7	13·0

in a concentration of 0.2 per cent (w/v) had any marked effect upon the blood haemoglobin concentration or female gonad weight.

The therapeutic effect of sulphamezathine or sulphapyrazine on coccidiosis is neutralized by *p*-aminobenzoic acid. The results shown in Table II demonstrate that neither the effect of sulphamezathine on the development of the testes nor on the prothrombin is neutralized by addition of *p*-aminobenzoic acid. The figures for body growth (Table III) show that sulphamezathine reduced the rate of body growth and that normal growth was not restored by addition of *p*-aminobenzoic acid. These results indicate that the effects of sulphamezathine on blood prothrombin and on body growth are not due to a reduction in vitamin synthesis owing to partial sterilization of the gut, although if *p*-aminobenzoic acid is more

TABLE III

AVERAGE WEIGHT IN GRAMS OF CHICKS AT 7-DAY INTERVALS

Treatment	No. of chicks	7 days (i.e., initial wt.)	14 days	21 days	28 days	35 days
Sulphamezathine	11	55·0	74·2	113·6	164·0	204·1
Sulphamezathine + *p*-aminobenzoic acid	12	56·2	80·8	118·3	162·5	211·9
p-aminobenzoic acid	12	54·6	97·7	144·8	226·9	277·6
Water control	12	54·9	97·3	149·0	229·7	275·8

completely absorbed than sulphamezathine the latter might still inhibit vitamin synthesis by intestinal flora ; it was, however, found that the administration of sodium succinyl sulphathiazole (0.2 per cent (w/v) of drinking water) had no effect on body growth, prothrombin time, or testes size of chicks.

TABLE IV

THE EFFECT OF VITAMIN K ON THE BLOOD PROTHROMBIN LEVELS OF CHICKS TREATED WITH SULPHAMEZATHINE

(*a*) *Groups of* 7*-day-old chicks under experiment for* 17 *days*

Food	Supplements	No. of chicks	Prothrombin time (sec.)	
			undil.	dil. × 4
Standard mash	None	8	23·3	39·4
Standard mash	Sulphamezathine (0·2%) in drinking water	8	32·9	55·5
Vitamin K deficient	None	6	53·5	82·2
Vitamin K deficient	Sulphamezathine (0·2%) in drinking water	6	46·1	71·8
Vitamin K deficient	Sulphamezathine (0·2%) in drinking water, menaphthone (10 mg. per kg.) in food	6	19·25	33·2
Vitamin K deficient	Menaphthone (10 mg. per kg.) in food	5	18·2	34·2

TABLE IV—*continued.*

(b) Groups of 8-day-old chicks under experiment for 15 days

Supplement	No. of chicks	Final body wt. (grams)	Prothrombin time		Testes weight (mg.)	
			undil.	dil. × 4	actual	mg./100 g. body wt.
Sulphamezathine, 0·2% sol.	6	125·5	36·8	54·7	28·4	21·9
Sulphamezathine, 0·2% sol. + menaphthone, 10 mg./kg. food	7	126·7	29·0	41·1	39·6	30·4
Menaphthone, 10 mg./kg. food	7	135·3	25·4	37·1	15·4	11·3
None	7	140·7	27·9	38·7	16·9	12·0

(c) Groups of 9-week-old cockerels under experiment for 26 days

Supplement	No. of chicks	Increase in wt. during expt. (g.)	Prothrombin time (sec.)	
			undil.	dil. × 4
Sulphamezathine, 0·2% sol.	10	353·6	71·1	124·6
Sulphamezathine, 0·2% sol. + synkavit 0·0025%	10	262·6	30·7	51·2
Sulphamezathine 0·3% + synkavit 0·0025%	10	257·9	30·1	56·6
None	6	388·7	33·1	60·8

The effect of sulphamezathine on blood prothrombin is similar to but not so great as that produced by vitamin K deficiency, and it was completely neutralized by the addition of menaphthone (vitamin K) to the diet (Table IVa); the effect of sulphamezathine on testes development, however, was not influenced by vitamin K (Table IVb). The effect of combining soluble vitamin K (calcium-2-methyl-1 : 4-dihydroxynaphthalinediphosphoric ether, "synkavit" Roche) and sulphamezathine in a single solution gave similar results (Table IVc). However, in a further experiment (Table VI, expt. E) three of a group of seven older chickens which received sulphamezathine (0.25 g. per kg. body weight daily), combined with vitamin K (menaphthone, 25 mg. per kg. body weight), showed intestinal haemorrhages on post-mortem examination; this indicates that haemorrhagic lesions in older birds may not be entirely due to deficiency of prothrombin.

Sulphamezathine has a rapid effect upon the blood clotting mechanism similar to that of dicoumarin (3 : 3′-methylenebis-4-hydroxycoumarin), the causative agent of sweet clover disease in cattle. Sulphamezathine appears to be quantitatively less effective than dicoumarin (Table V) because a single dose of 2 g. per kg. body weight sulphamezathine had less effect on prothrombin time than 150 mg. per kg. dicoumarin. The effect of sulphamezathine on prothrombin level disappears between four and ten days after the withdrawal of sulphamezathine.

Although the effect of sulphamezathine on blood clotting develops rapidly, the addition of varying amounts of sodium sulphamezathine to plasma *in vitro* was found to have no effect on the prothrombin time. Sulphamezathine had no

TABLE V

THE EFFECT OF LARGE DOSES OF SULPHAMEZATHINE AND DICOUMARIN ON PLASMA PROTHROMBIN OF 23-DAY-OLD CHICKS

Prothrombin times (sec.)

Sulphamezathine, 3 doses of 1 g./kg. body wt. 42, 26 and 18 hr. before sampling		Sulphamezathine, single dose of 2 g./kg. body wt. 18 hr. before sampling		Dicoumarin, 150 mg. per kg. body wt. 18 hr. before sampling		Normal control	
undil.	dil. × 4	undil.	dil. × 4	undil.	dil. × 4	undil.	dil. × 4
24·2	61·0	39·4	68·8	48·0	113·8	19·2	44·6
27·2	69·4	44·2	110·4	33·8	78·4	24·4	49·6
26·4	58·2	46·8	87·6	54·4	112·2	25·0	36·4
28·6	58·4	29·0	61·4	43·0	118·2	30·4	58·8
		30·8	69·0			25·2	42·0
Mean 26·6	61·75	38·0	79·4	44·8	105·7	24·8	46·3

direct anticoagulant action similar to that of heparin or of dyes such as chlorazol fast pink ; the effect on blood clotting *in vivo* may therefore be independent of the actual concentration of the drug at the time when the blood is taken.

Haemorrhagic lesions in chicks

A proportion of chicks dosed with sulphamezathine have developed multiple haemorrhages, while no such haemorrhages have been seen in a large number of control chicks, or in chicks treated with other sulphonamides. It is therefore clear that this syndrome is induced by sulphamezathine, and the experiments described suggest that the action is similar to that of sweet clover in producing haemorrhagic disease in cattle. In various experiments a total of ninety-one 7-day-old chicks were given a 0.2 per cent (w/v) solution of sulphamezathine for periods of 21–35 days ; intestinal haemorrhages were present in 6 cases only and there were no deaths attributable to haemorrhage. In all chicks of this group lesions were confined to the intestines ; they varied from very numerous petechiae extending throughout the greater part of the length of the intestine to relatively few larger haemorrhages of 3–5 mm. diameter. The haemorrhages were readily visible through the serosa. The lesions occurred in all layers of the bowel wall, but were most frequent in the submucosa.

Chickens treated for caecal coccidiosis on farms are usually between 6 and 12 weeks old. All field cases of sulphamezathine poisoning, characterized by haemorrhagic lesions, which have been brought to our attention have occurred in chickens of this age.

The incidence of haemorrhagic lesions shown in Table VI indicates that chickens of 6 weeks old are more susceptible to the haemorrhagic effect of sulphamezathine than are younger chicks ; for instance, in experiment E, 7 out of 13 chickens killed after 3 weeks' treatment with 0.25 g. sulphamezathine per kg.

TABLE VI

THE EFFECT OF ADDITION OF SULPHAMEZATHINE TO DRINKING WATER OF 6–12-WEEK-OLD CHICKENS

Exper.	No. of chickens	Age	Dose	Period of treatment	Effect
A	8	7 days	0·2% sol.	100 days	Two died from haemorrhage on 44th and 50th days. Six survived and remained healthy
B	9	9 weeks	0·2% sol.	97 days	None died. Precocious development of comb and wattles and male plumage
C	11	6 weeks	0·2% sol.	62 days	Four died from haemorrhage on 18th, 36th and 39th days; two killed on 63rd day showed small intestinal haemorrhages. Five normal
D	15	6 weeks	0·2% sol.	70 days	Three died, 26th, 44th and 56th days, with haemorrhages: four killed showed haemorrhages. Eight normal
E	7	6 weeks	0·25 g. per kg. body wt. daily	20 days	None died. Four showed haemorrhages when killed on 21st day
	6	6 weeks	0·25 g. per kg. body wt. daily + menaphthone 25 mg. per kg. body wt. daily	20 days	None died. Three showed internal haemorrhages when killed on 21st day
F	7	10 weeks	0·2% sol.	15 days	No deaths or gross lesions
	6	10 weeks	0·2% sol. + synkavit 50 mg. per l.	15 days	No deaths or gross lesions

body weight daily were found to possess intestinal haemorrhages. Furthermore, 9 out of the 43 chickens receiving 0.2 per cent (w/v) sulphamezathine for 62–100 days (experiments A, B, C, and D) died as the result of internal haemorrhage. Most of the chickens remained in apparently good health after prolonged administration of sulphamezathine, but post-mortem examination of them frequently revealed intestinal haemorrhages similar to those of younger chicks. The lesions in chickens dying as a result of haemorrhage were widespread and severe. Haemorrhages were invariably present in the intestinal tract; the liver also was frequently bespattered with haemorrhagic foci. Massive haemorrhages were seen in the skeletal muscles, particularly in the muscle groups of the thighs and breasts. The ventral border of the gizzard was the site of haemorrhage in two fowls. Persistent bleeding from feather follicles resulted in the deaths of two chicks. It appeared that haemorrhages might occur at any site, but were most frequent at those points particularly exposed to trauma.

Testicular hyperplasia

Precocious sexual development of cockerels was often evident after 10–14 days' dosing with sulphamezathine. With continued dosing, growth of combs and wattles proceeded rapidly, so that after 4 weeks a stage of development normally seen in cockerels of 2–3 months old was reached.

The development of secondary sexual characters was evident in only a proportion of the dosed cockerels. Macroscopic enlargement of testes was invariably present in chicks showing obvious comb and wattle development. Of the 71 chicks receiving sulphamezathine for 21–28 days, 43 had enlarged testes, 21 were approximately normal in size, and 7 were abnormally small. The testes weights of some sulphamezathine-treated chicks after 4 weeks were as much as five times greater than those of untreated control chicks. Sulphamezathine regularly inhibited the body growth of chicks (Tables I and III) and in general it was the chicks which had the best growth rates which developed testicular hyperplasia. A proportion of treated chicks grew very poorly, and in these the testes were very small and inactive.

Sulphamezathine-treated chicks which responded with testicular hyperplasia had haemoglobin and prothrombin levels not significantly different from those of chicks failing to respond in this way (Table VII).

Quantitative data on the enlargement of the testes of cockerels has been given. Table I shows that there was no comparable enlargement of the female gonad after dosing for 4 weeks with any of the sulphonamides tested, and microscopic examination revealed no abnormalities. Sulphamezathine has more effect on the testes than either sulphamerazine (sulphamethylpyrimidine) or sulphadiazine (sulphapyrimidine). Although the average testes weights of chicks dosed with sulphadiazine (Table I) do not indicate enlargement, microscopic examination revealed that the testes of some treated chicks were actively stimulated. Sulphathiazole, succinyl sulphathiazole, and sulphapyrazine had no such action.

The effect was not neutralized by simultaneous dosing of an amount of *p*-aminobenzoic acid which completely neutralized the therapeutic action of the drug in caecal coccidiosis (Horton-Smith and Boyland, 1946 ; cf. Table II) ; nor was it neutralized by menaphthone (Table IVb).

Microscopical examination of sections of the cockerel testes showed that the main action of the drug was on the seminiferous tubules which were hyperplastic (Figs. 2 and 3). The interstitial tissue remained scanty and appeared to be compressed by the proliferating tubules, but active interstitial tissue was present and this was the probable source of the male hormone. The enlarged seminiferous tubules were lined with spermatocytes in active mitotic division. In the lumen of the tubules there were degenerative forms so that the centre of the tubules sometimes contained a "necrotic area." Sometimes (as in Fig. 4) the hyperplasia occurred in a few tubules, whilst the other tubules remained almost normal and immature.

TABLE VII

A COMPARISON OF THE BODY WEIGHTS, PROTHROMBIN TIMES AND HAEMOGLOBIN LEVELS OF COCKEREL CHICKS WHICH RESPONDED TO SULPHAMEZATHINE DOSING BY PRECOCIOUS SEXUAL DEVELOPMENT, AND THOSE FAILING TO DO SO

Group of cockerels	Body weight (grams)	Testes weight mg./100 g. body weight	Prothrombin clotting times		Haemoglobin g. per 100 ml.
			undil.	dil. × 4	
Dosed sulphamezathine Good body growth Marked comb development	295	39·6	51·2	73·4	7·84
Dosed sulphamezathine Poor growth No comb development	166	8·5	48·7	68·6	7·54
Control—undosed ..	308	13·6	34·8	49·6	7·58

The stimulus to sexual development provided by sulphamezathine to cockerels disappeared upon withdrawal of the drug, so that it became impossible to distinguish between dosed and undosed cockerels 3 weeks after the end of the treatment.

Extracts of testes from normal and sulphamezathine-treated cockerels appeared to have no hyaluronidase activity. This is perhaps to be expected, as the immature testes contained no mature spermatozoa.

Microscopic examination of the thyroid glands of the sulphamezathine-dosed chicks failed to reveal any change.

The effects of certain dimethylpyrimidines on cockerel chicks

Only those sulphonamides which contain a pyrimidine ring, i.e., sulphamezathine, sulphamerazine, and sulphadiazine, appear to have any action on the testes. Sulphamezathine, which contains a dimethylpyrimidine ring, was much more active in this respect than either sulphamerazine or sulphadiazine. Groups of 7-day-old chicks were dosed with 0.2 per cent (w/v) solutions of 2-hydroxy-4:6-dimethylpyrimidine (white form), 2-hydroxy-4:6-dimethylpyrimidine (yellow form), 2-amino-4:6-dimethylpyrimidine, and 2-mercapto-4:6-dimethylpyrimidine in place of drinking water. None of these substances had any effect on testes size or prothrombin time. The mercapto compound reduced the rate of growth, but the hydroxy compounds and the amino derivative had no effect on body growth.

The effect of sulphamezathine on drakelets

A group of ten 8-day-old drakelets was dosed with 0.2 per cent (w/v) sulphamezathine in their drinking water for 28 days. Dry food of the same composition as used for chicks was made into a damp mash with sulphamezathine solution. The sulphamezathine was readily consumed. After 28 days' treatment all drakelets were killed and examined. There was no evidence of intestinal haemorrhages in any of the birds. The treatment reduced

the growth rate, but had no effect on testes weight or on prothrombin and haemoglobin levels. The sulphonamide level of the blood of ducklings dosed with 0.2 per cent (w/v) sulphamezathine (4.4 mg. per 100 ml.) was lower than that of chicks receiving the same dose.

The effect of sulphamezathine on the sex organs of male rats

Groups of ten rats weighing 50–60 g. were placed on diets containing sulphonamide (as 0.1 per cent of the dry matter of the diet). Half the rats in each group were castrated just before being put on the diets. The rats were weighed at the beginning of the experiment and at intervals throughout the period of dosing. The data (Table VIII) show that growth was considerably inhibited by sulphapyrazine—rats on this drug hardly grew at all during the last two weeks of treatment—while sulphathiazole had no, and sulphamezathine only a slight, inhibitory effect on growth.

TABLE VIII

THE EFFECT OF DOSING SULPHONAMIDES FOR 34 DAYS ON SEX ORGANS OF MALE RATS

Nature of animals	Mean body wt., g.		Testes, mg. mean	Seminal vesicles and prostate, mg. mean
	Beginning	End		
Undosed normal rats	64	143	850	272
Undosed castrated rats	66	146	—	90
Normal rats dosed with sulphamezathine	55	119	880	426
Castrated rats dosed with sulphamezathine	63	127	—	88
Normal rats dosed with sulphapyrazine	53	71	480	79
Castrated rats dosed with sulphapyrazine	58	86	—	90
Normal rats dosed with sulphathiazole	56	139	960	292
Castrated rats dosed with sulphathiazole	59	135	—	85

None of the drugs produced any marked enlargement of the testes of normal rats, or enlargement of the seminal vesicles and prostate of castrated rats. Sulphamezathine thus has no male hormone action on adult rats and does not cause testicular hypertrophy. On the other hand, the seminal vesicles and prostate of normal rats treated with sulphamezathine were larger, and those of rats treated with sulphapyrazine smaller, than the same organs of rats fed on normal diet or on a diet containing sulphathiazole. Thus although sulphamezathine had no effect on castrated rats and very little on the weight of testes of normal rats, it caused enlargement of the seminal vesicles and prostate presumably by stimulation of the testes.

When rats were dosed with sulphamezathine from birth, hyperplasia of the testes occurred which was obvious in rats weighing less than 50 g. Pregnant rats were fed on a diet containing 0.1 per cent sulphamezathine and the diet was given to the mother and young after birth. The weights of the testes of young rats of different ages dosed with sulphamezathine are compared with the weights of normal animals in Table IX. While the testes weight per 100 g. body weight increases from 380 to 700 mg. in normal rats from 16 to 52 g. in weight, the weights vary from 600 to 1300 mg. per 100 g. body weight in sulphamezathine-dosed rats from 17 to 54 g. body weight. Increases in size of seminal vesicles and prostate occurred in young rats, but these were less than the increases in size of these organs in adult rats dosed with sulphamezathine. The increase in testicle size (Figs. 5 and 6) is similar to the effect on the cockerel testes.

TABLE IX

THE WEIGHT OF SEX ORGANS OF YOUNG NORMAL MALE RATS AND YOUNG RATS DOSED WITH SULPHAMEZATHINE FROM BIRTH

A. Normal Rats

Wt. of rat, g.	16	22	29(2)	32(4)	46(2)	52(2)
Wt. of testes, mg.	61	97	120	163	303	363
Wt. of seminal vesicles and prostate, mg.	30	32	47	55	66	71
Testes wt. as mg./100 g. body wt.	380	440	410	510	660	700
Seminal vesicle and prostate wt. as mg./100 g. body wt. ..	187	146	162	172	144	196

B. Rats Dosed with Sulphamezathine

Wt. of rats, g.	17	29(2)	35	38	47(2)	50(2)	54
Wt. of testes, mg.	103	221	302	330	609	585	702
Wt. of seminal vesicles and prostate, mg.	34	48	65	99	94	104	93
Testes wt. as mg./100 g. body wt.	600	760	860	870	1290	1170	1300
Seminal vesicle and prostate wt. as mg./100 g. body wt. ..	200	165	186	260	200	204	173

Some figures are averages of groups of rats of the same weight. The number in such groups is indicated in parentheses.

The toxicity of sulphamezathine in adult fowls

Two groups of six mature R.I.R. pullets and cockerels were dosed by mouth with a 16 per cent (w/v) solution of sodium sulphamezathine at a rate of 0.25 g. per kg. body weight daily for 14 days. The pullets which had been in full production ceased to lay on the 3rd day. The fowls became sick and deaths of pullets occurred on the 6th, 7th, 9th, and 16th days, and of cockerels on the 7th, 12th, 13th, 15th, and 16th days. The surviving cockerel remained apparently normal and when killed on the 21st day showed no apparent lesions. The two surviving pullets were very sick on the 15th day but improved by the 21st day, when they were killed. Both had low haemoglobin concentrations (7.5 and 8.1 g. per 100 ml.). On post-mortem examination both were found to have retained fully formed eggs in the oviduct; the eggs were coated with a fibrinous deposit and appeared to have remained stationary for some time.

Obvious symptoms were only apparent during the four days prior to death: although appetite was decreased, the crop remained distended with food; the feathers around the vent were matted and stained with yellow faecal material; the excreta of some birds had a bright greenish-yellow colour which resembled that of fowls affected with fowl typhoid. Some fowls were disinclined to move about and locomotor ataxia was apparent for about 24 hours before death. The colour of the comb and wattles changed in some instances to a bright salmon-red; in one fowl the head appendages became blue and shrivelled. Body temperatures were elevated up to a maximum of 110.4° F. On examination of the cloacal mucosa petechiae could sometimes be observed. In cockerels the genital eminences became green or brown as a result of extravasated blood.

Post-mortem examination revealed widespread lesions. Haemorrhages were invariably present in the intestinal tract, extending from the duodenum to the cloaca (Fig. 7); the gizzard lining of one fowl also was involved. The haemorrhages were similar to those seen in younger chickens and varied in size from petechiae to plaques of 5 mm. diameter. The lesions in the large intestine became ulcerated as a result of erosion of the mucosa; the contents of the intestines were mucoid and sometimes bloodstained; petechiae were present in the serous membranes, in the abdominal fat, at the base of the heart, and of the gizzard. The liver was somewhat enlarged and friable; large wedge-shaped infarcts were seen; the

colour of the liver of two fowls was changed to green. The kidneys were enlarged and the colour changed as in the liver. Numerous basophilic erythrocytes appeared before death and the haemoglobin level fell sharply. The spleen was slightly enlarged in some cases, and contained subcapsular haemorrhages. The ovarian follicles were soft and flabby, and in one case yolk material was present in the peritoneal cavity. A coliform organism was isolated in pure culture from the spleens of three fowls and heart blood of one. It is probable that the infecting organisms invaded the blood from the gut through the intestinal lesions. Similar effects on the gut were produced when sulphamezathine (250 mg./kg. body weight per day) was administered by feeding in capsules (cf. Fig. 8).

The effect of sulphamezathine on the breeding capacity of fowls

Four 10-month-old R.I.R. cockerels were dosed with 0.15 g. per kg. body weight daily. Examination of semen collected from these cockerels, before dosing commenced and at intervals during treatment, failed to reveal any change in the yield of semen, or the density, form, or motility of sperms. One cockerel died after the 36th dose with lesions of sulpha-

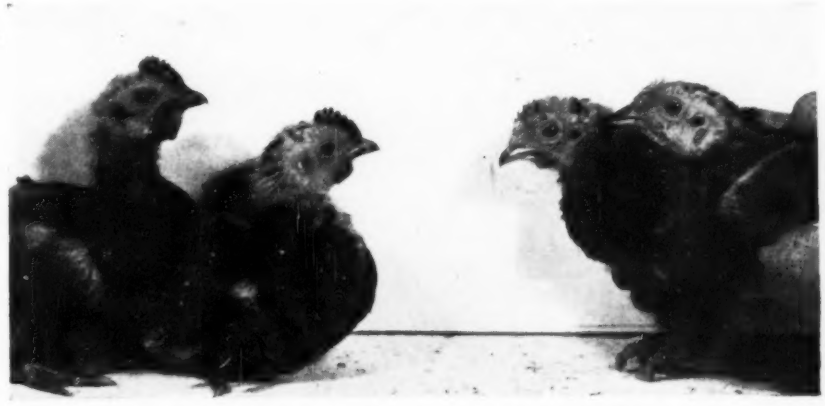


FIG. 1.—35-day-old cockerels. The two on the left were dosed with sulphamezathine for the last 28 days and show enlarged combs and wattles as compared with the two controls on the right.

mezathine poisoning. After the 50th dose the cockerels were mated to three pens (A, B and C) each of six virgin pullets. The cockerel in pen A died on the 58th day of dosing from sulphamezathine poisoning. Fourteen eggs laid by pullets in pen A between the 53rd and 58th day were incubated: 5 were fertile and 3 hatched normal chicks. Dosing of the cockerels in pens B and C continued until 102 doses had been given but had no apparent effect upon fertility.

A group of six laying hens and a cockerel were dosed with 0.2 per cent (w/v) sulphamezathine in their drinking water. Egg production ceased by the 3rd day, 2 eggs were laid on the 10th day; all six hens had resumed laying by the 15th day. During the succeeding 6 weeks 42 eggs were incubated of which 5 were infertile; 28 normal chicks were hatched from the remaining 37 fertile eggs. Thus the dosing had only a temporary effect on the egg-laying of fowls.

DISCUSSION

As sulphamezathine and sulphapyrazine are so useful in treatment of coccidiosis in chickens it is of value to know what other effects these drugs may have.

FIG. 2.—Section of testis from normal 35-day-old cockerel. H and E ×660.

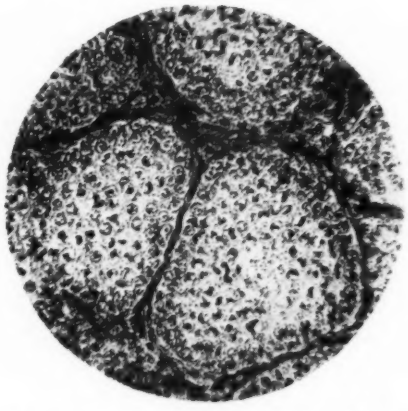


FIG. 3.—Section of testis from 35-day-old cockerel, dosed with sulphamezathine (0.2% in drinking water) for 28 days, showing hypertrophy of tubules. H and E×660.

FIG. 4.—Section of testis from 35-day-old cockerel dosed with sulphamezathine showing hypertrophy of part of the tubular tissue while other tubules are not enlarged. H and E×100.

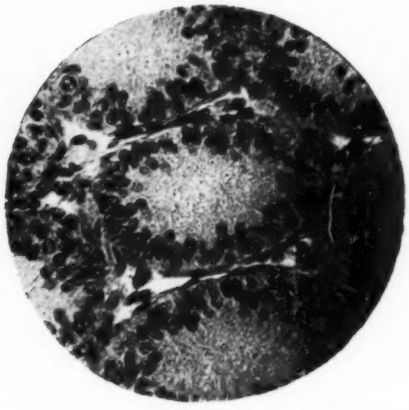


FIG. 5.—Section of normal testis from rat weighing 16g. H and E×660.

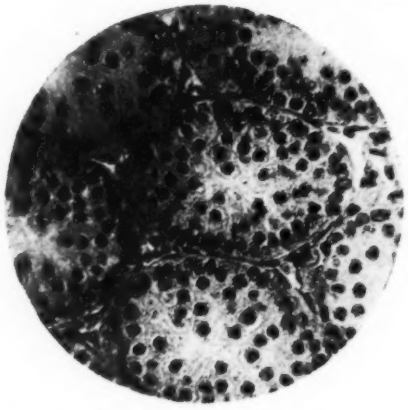


FIG. 6.—Section of testis from a rat weighing 17 g. and dosed with sulphamezathine from birth. H and E×660.

While sulphamezathine is liable to cause hyperplasia of the testes of young cockerels, lengthens the blood clotting time, reduces the rate of growth, and causes haemorrhage in the gut, sulphapyrazine has not been found to have any of these effects. Sulphapyrazine is also quantitatively more effective than sulphamezathine in curing coccidiosis, but it is not prepared and is not available in England. In spite of the pathological actions which sulphamezathine can have, it remains a valuable drug because the toxic effects are not produced unless treatment is carried on for longer than the recommended period of 7 days. Prolonged treatment has, however, been used by some farmers and a few deaths from haemorrhage have occurred.

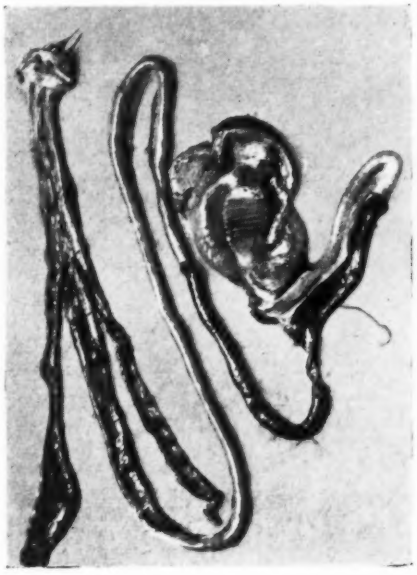


FIG. 7.—Alimentary tract from chicken dosed with sulphamezathine showing widespread haemorrhagic lesions.

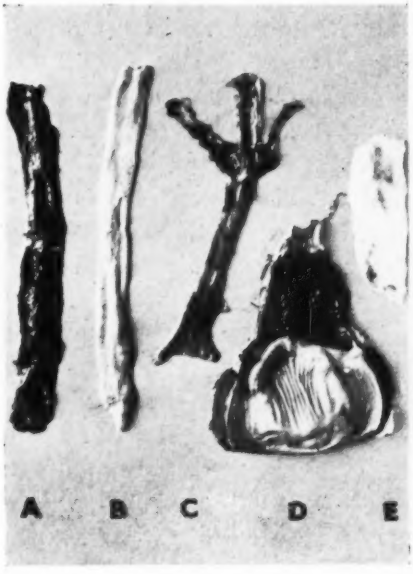


FIG. 8.—(*a*) Duodenum from chicken dosed with sulphamezathine. (*b*) Corresponding duodenum from normal chicken. (*c*) Rectum and caecum from chick treated with sulphamezathine. (*d*) Gizzard and proventriculus from chicken treated with sulphamezathine. (*e*) Omental fat with haemorrhages from chicken treated with sulphamezathine.

The increase in therapeutic effect, paralleled by an increase in effects on the blood clotting system and on the testes, which occurs when one (sulphamerazine) or two (sulphamezathine) methyl groups are introduced into the sulphadiazine molecule, may be due in part to the increased absorption of these methyl sulphapyrimidines.

The effect on blood clotting, and probably to some extent on induction of haemorrhages, can be neutralized by feeding menaphthone or by the addition of a soluble vitamin K preparation to the sulphamezathine solution. In view of this it might be advisable to incorporate a small amount of a vitamin K preparation in sulphamezathine solutions issued for treatment of caecal coccidiosis.

Summary

1. Sulphamezathine, which is an excellent drug for the treatment of caecal coccidiosis in chickens, has certain undesirable side effects. These effects include : (*a*) a decrease in the rate of clotting of the blood, similar to that produced by dicoumarin, which is neutralized by vitamin K ; (*b*) induction of haemorrhages, particularly in chicks between 6 and 12 weeks old dosed for several weeks ; (*c*) a decrease in rate of growth ; and (*d*) a hyperplastic action on the testes of young cockerels, accompanied by growth of the comb and wattles and precocious sexual development.

2. Sulphapyrazine, which is somewhat more effective than sulphamezathine in treatment of coccidiosis, has none of these effects.

3. Sulphamezathine causes hyperplasia of the testes of young rats, but not of adult rats. The increase in testes size is accompanied by some increase of the seminal vesicles and prostate. The drug has no effect on the seminal vesicles and prostate of castrated rats.

4. Sulphamezathine does not appear to affect the fertility of adult cockerels or the laying capacity of fowls.

We wish to thank Dr. P. Koller for preparing the microphotographs, Mr. D. M. Brown and Dr. F. Rose for gifts of the dimethylpyrimidine derivatives, and Dr. F. Bergel, of Messrs. Roche Products, for synkavit.

References

Asplin, F. D., Boyland, E., and Horton-Smith, C. (1946). *Biochem. J.*, **40**, ii.
Horton-Smith, C., and Boyland, E. (1946). *Brit. J. Pharmacol.*, **1**, 139.
Horton-Smith, C., and Taylor, E. L. (1943). *Vet. Record*, **55**, 109.
Witts, C. J., and Hobson, F. C. G. (1940). *Brit. med. J.*, **2**, 862.

Brit. J. Pharmacol. (1947), **2**, 93.

THE COMPARISON OF RESPIRATORY STIMULANT DRUGS

BY

R. H. THORP

From the Wellcome Physiological Research Laboratories, Beckenham, Kent

(Received December 11, 1946)

Drugs stimulating respiration are usually classified as analeptics or as respiratory analeptics. This latter term is perhaps more useful since the term "analeptic" has such a wide significance that, in addition to picrotoxin and leptazol, it can also include such substances as amphetamine and ephedrine, which have an action at higher levels in the central nervous system. The method about to be described has been devised primarily for the more purely respiratory type of analeptic.

The methods usually employed to compare analeptics (using the term generally) are based upon the reduction they produce in the duration of anaesthesia. Trevan (1939) used this principle to compare the central stimulant actions of amphetamine and isomers of ephedrine in mice which had been anaesthetized with paraldehyde. He also included some experiments in which picrotoxin and leptazol were employed as the stimulant drugs, which showed that amphetamine had a greater awakening effect upon mice than either picrotoxin or leptazol, but that leptazol is slightly more effective than picrotoxin.

This type of method is very suitable for the examination of compounds of the amphetamine type in which Trevan was primarily interested, but has several disadvantages when employed to test the more predominantly respiratory stimulants.

Chakravati (1939) pointed out some of the objections to this method and attempted to overcome them. One of the main objections is the variable depth of anaesthesia resulting from the administration of a fixed dose of anaesthetic to a series of mice, and Chakravati attempted to sort out his animals according to the depth of anaesthesia. Despite this, several objections still remain. Firstly, the animals are not necessarily anaesthetized to a constant depth and, secondly, the degree of anaesthesia is not comparable with that occurring in patients when analeptics are required. When these drugs are used clinically it is usually to combat excessive degrees of anaesthesia resulting, most often, from barbiturate anaesthetics.

The criterion by which stimulant drugs will be judged in such clinical use is not so much the extent to which they will awaken the patient as the reliability with which respiration can be improved with their aid. The two effects are interrelated since the awakening effect will follow the use of a successful respiratory stimulant more quickly than if it were not employed.

A most interesting contribution was made to the study of analeptic drugs of this type by Das (1939), who gave continuous intravenous injections of anaesthetics to depress the respiration of rabbits by about 50 per cent and then observed the effects of different stimulant drugs upon that level of anaesthesia. The method we have used is similar to that of Das except that a much more critical level of anaesthesia is employed.

Experimental Procedure

Guinea-pigs weighing between 250 and 700 g. are used, in groups of eight or ten animals, the weight distribution in any set of animals being not more than ± 20 per cent different from the mean value for the group. The animal is secured prone on an operating table and the hair is removed from the outer surface of the forelimbs between the ankle and the knee. A bleb is raised on each forelimb by the injection of 0.1 c.c. of a solution of 2 per cent (w/v) procaine subcutaneously just over the route of the accessory cephalic vein. After a minute the skin in this region is plucked up with forceps and a small patch cut out with scissors, thus exposing about ¼ in. of the vein in each forelimb.

The anaesthetic is made up in such a strength that it will kill the guinea-pig when infused continuously in about ten minutes. The anaesthetics we have used are thiopentone soluble and pentobarbital soluble, both made up in solutions of 7.5 mg./c.c. The rate of continuous injection employed does not seem very important, but for these solutions 0.6 c.c./min. has been used and found satisfactory.

The anaesthetic solution is injected into the animal by means of a fine dental needle inserted in the vein and attached to a motor-driven syringe containing the solution. With a little practice this injection becomes a simple matter. As injection proceeds the depth of anaesthesia increases until there is considerable respiratory depression. The intervals between successive breaths become longer and longer. When twenty seconds have elapsed after a breath the injection is stopped. At this level of anaesthesia spontaneous recovery is very improbable. If the animal is kept warm, however, the heart will continue to beat for several minutes. The analeptic drug is next injected into the vein of the opposite limb in a similar manner, as a solution containing a half to one LD50 of the substance per c.c. In our experiments a rate of 0.6 c.c./min. was employed.

A true respiratory analeptic will cause respiration to start again in a few minutes and the amount of drug required to do this is recorded. The injection of the drug is continued until there are signs of over-stimulation, such as convulsive jerks; the injection is then stopped and the total amount of the analeptic drug given is recorded.

The guinea-pig is now placed, with the cuts on the forelimbs protected by pads of absorbent cotton wool, in a constant temperature cabinet at 30° C. and observed, until it is clear whether the animal will live or die. If the animal lives for two hours death later is very unlikely; animals surviving this period are then destroyed.

We have termed the dose required to restart respiration, and the dose required to produce over-stimulation, *A* and *B* respectively. The values of the *A* and *B* doses per unit weight can be compared for different analeptics.

Results

Experiments were first made to determine for how long the injection of the anaesthetic must be continued after the cessation of respiration in order to avoid spontaneous recovery. The results obtained are given in Table I and indicate

TABLE I

THE EFFECT OF CONTINUING THE INJECTION OF PENTOBARBITAL SOLUBLE (4·5 MG./MIN.) AFTER THE LAST BREATH UPON THE MORTALITY OF GUINEA-PIGS

Time lapse after the last breath in seconds	Mortality after 1 hour
0	1/10
5	2/10
10	8/10
20	10/10
20	10/10
20	10/10

that such recovery is very unlikely when twenty seconds have elapsed after the last breath. Table II shows the mean doses of the two anaesthetics required to cause respiratory arrest in seven groups of ten guinea-pigs for each anaesthetic, together with the standard errors of these mean values. The two final means

TABLE II

MEAN DOSES OF ANAESTHETIC REQUIRED TO CAUSE PERMANENT RESPIRATORY ARREST IN GROUPS OF TEN GUINEA-PIGS. BOTH ANAESTHETICS WERE INJECTED AT 4·5 MG. PER MINUTE INTRAVENOUSLY

Thiopentone soluble			Pentobarbital soluble		
Group of 10 animals	Mean dose mg./kg.	Standard error	Group of 10 animals	Mean dose mg./kg.	Standard error
1	46·0	1·8	8	68·6	6·0
2	46·7	2·9	9	63·3	4·6
3	44·9	2·5	10	73·6	4·8
4	41·0	3·8	11	68·0	3·7
5	45·2	0·2	12	69·1	2·1
6	50·4	3·2	13	65·5	2·9
7	36·9	1·5	14	64·4	1·7
Mean	44·44	1·04	Mean	67·50	1·6

give an estimate of the LD50 of thiopentone soluble and pentobarbital soluble in guinea-pigs. Each group of ten guinea-pigs was treated on the same day, but the fourteen groups were used over a period of several weeks. It will be seen that the groups of animals give consistent results one with another and the standard errors within each group are small.

For the assessment of a respiratory stimulant drug two groups of ten animals were used, one with each of the anaesthetics, which were chosen to give both short and long durations of anaesthesia ; thiopentone soluble was chosen for short and pentobarbital for long durations. The former drug would be more readily antagonized and the latter, owing to its longer action, would provide a better test of the analeptic property.

The results obtained with picrotoxin are given *in extenso* in Table III and the corresponding mean results for leptazol and triazol 156 in Table IV. Triazol

TABLE III

TYPICAL SET OF RESULTS OBTAINED BY THE METHOD DESCRIBED SHOWING THE RELATIVE RESPIRATORY STIMULANT ACTIONS OF PICROTOXIN UPON THE RESPIRATION DEPRESSED WITH THIOPENTONE AND PENTOBARBITAL. *A* IS THE DOSE TO RESTART RESPIRATION AND *B* THE DOSE TO CAUSE OVER-STIMULATION

Thiopentone anaesthesia				Pentobarbital anaesthesia			
Animal No.	Picrotoxin dose mg./kg.		Result of observation for 2 hr.	Animal No.	Picrotoxin dose mg./kg.		Result of observation for 2 hr.
	A	*B*			*A*	*B*	
1	1·4	2·5	Lived	11	10·4	14·4	Lived
2	2·0	2·8	Lived	12	9·8	14·1	Lived
3	2·4	10·8	Lived	13	5·2	11·8	Lived
4	2·8	6·8	Lived	14	6·0	11·5	Lived
5	2·5	7·6	Lived	15	5·8	11·9	Lived
6	—	—	Failed to recover	16	1·6	17·4	Lived
7	6·0	13·7	Lived	17	5·5	15·0	Lived
8	6·3	14·1	Lived	18	7·4	17·7	Died
9	5·0	6·8	Lived	19	5·3	12·7	Lived
10	4·8	10·4	Lived	20	5·6	17·8	Died
Mean	3·5	8·3	9/10 lived	Mean	6·28	14·43	8/10 lived
Std. error	0·183	1·40		Std. error	0·92	0·79	

156 or *cyclo*hexyl-ethyl-triazol, also known as "azoman," was described by Behrens, Dinkler, and Woenckhaus (1937). This drug resembles leptazol in many respects, but is much more potent.

The results can also be represented graphically as shown in Figs. 1 and 2. These graphs were obtained by plotting the logarithm of the dose required by any one guinea-pig either to restart respiration (*A* curves) or to cause over-stimulation (*B* curves) against the probit value for the percentage of the total number of animals which responded to that dose of the stimulant drug or to smaller doses. The mean value corresponds statistically to the LD50 in a toxicity test and causes recovery in 50 per cent of the animals. The relative doses of the analeptics required can be seen from the graphs ; the steepness of the curves gives a measure of the efficiency with which the drugs antagonize the anaesthetics. In Table IV the ratios of the potencies of the three analeptics are given,

taking picrotoxin doses as 1.0. It will be seen that, with thiopentone anaesthesia, both the *A* and the *B* values for triazol 156 bear a constant ratio of 0.47 to the corresponding values for picrotoxin. This is because both drugs cause over-stimulation by the same proportional increase in the doses required to restart the respiration. This is not so with pentobarbital anaesthesia: picrotoxin causes over-stimulation quite readily, but triazol gives a very much smaller ratio for over-stimulation because it does not antagonize this long-acting barbiturate as readily as picrotoxin does.

TABLE IV

A COMPARISON OF THREE ACCEPTED ANALEPTICS. THE VALUES UNDER *A* GIVE THE MEAN DOSES REQUIRED TO RESTART RESPIRATION AND UNDER *B* TO CAUSE OVER-STIMULATION. THE RATIOS ARE THE INVERSE RATIOS OF THE DOSES, TAKING PICROTOXIN AS 1.0.

Analeptic drug	Thiopentone anaesthesia						Pentobarbital anaesthesia					
	A		*B*		No. alive	Notes	*A*		*B*		No. alive	Notes
	Dose mg./kg.	Ratio	Dose mg./kg.	Ratio			Dose mg./kg.	Ratio	Dose mg./kg.	Ratio		
Picrotoxin .. 2.0 mg./c.c. 0.6 c.c./min.	3.5	(1.0)	8.3	(1.0)	9/10	*a*	6.3	(1.0)	14.4	(1.0)	8/10	*b*
Triazol 156 .. 4.0 mg./c.c. 0.6 c.c./min.	7.5	0.47	17.8	0.47	10/10	*c*	6.0	1.05	46.4	0.31	4/10	*d*
Leptazol .. 100 mg./c.c. 0.6 c.c./min.	218.0	0.016	—	—	9/10	*e*	234.0	0.027	—	—	4/8	*f*

(*a*) Muscular twitching persisted for 5–10 min.
(*b*) Slight muscular twitching after injection.
(*c*) Very smooth respiration without twitching. Quick awakening.
(*d*) Difficult to produce over-stimulation.
(*e*) No over-stimulation possible. Awakening effect most marked.
(*f*) No over-stimulation possible.

It will be seen from Table IV and Figs. 1 and 2 that the most effective analeptic available to antagonize the barbiturate anaesthetics employed is picrotoxin, but that triazol 156 is not greatly inferior. Picrotoxin is effective against both the short- and long-acting barbiturates used, but leptazol is much less effective against the long-acting drug. Triazol 156 is intermediate between the two and antagonizes pentobarbital far less easily than it does thiopentone anaesthesia. There is much more variability in the results giving the *B* curve for triazol and pentobarbital anaesthesia, and hence the slope of the curve is lower.

Leptazol is the least effective of these stimulant drugs and it did not cause over-stimulation in these experiments. If the injection is continued for a prolonged period, however, secondary depression occurs; when this was observed the injections were stopped one minute after respiration had been re-established.

This secondary depression has also been observed by Das (1939). When leptazol is effective, however, it reduces the anaesthetic duration most markedly. Animals frequently appeared quite conscious within about ten minutes of injecting the drug, although a relapse into deep anaesthesia was common, especially when

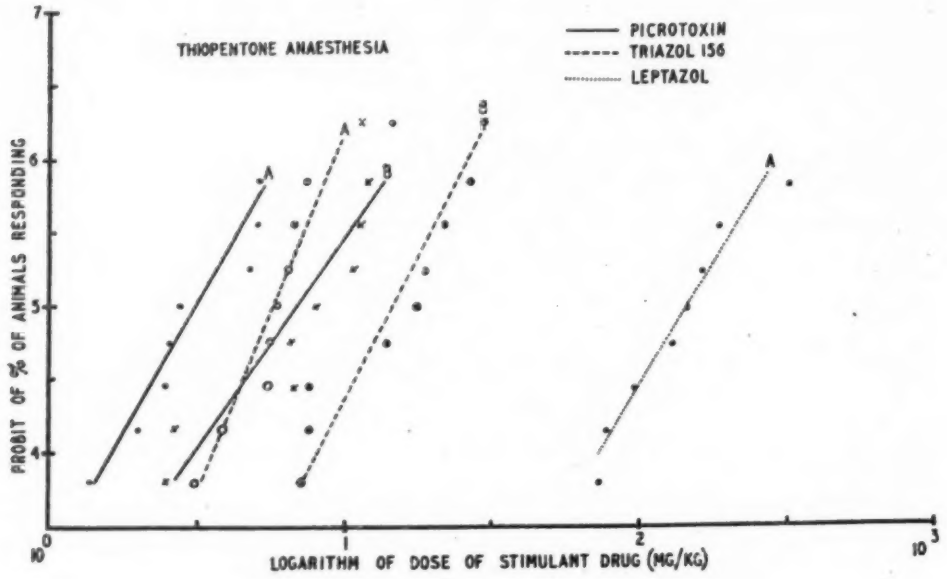


FIG. 1.

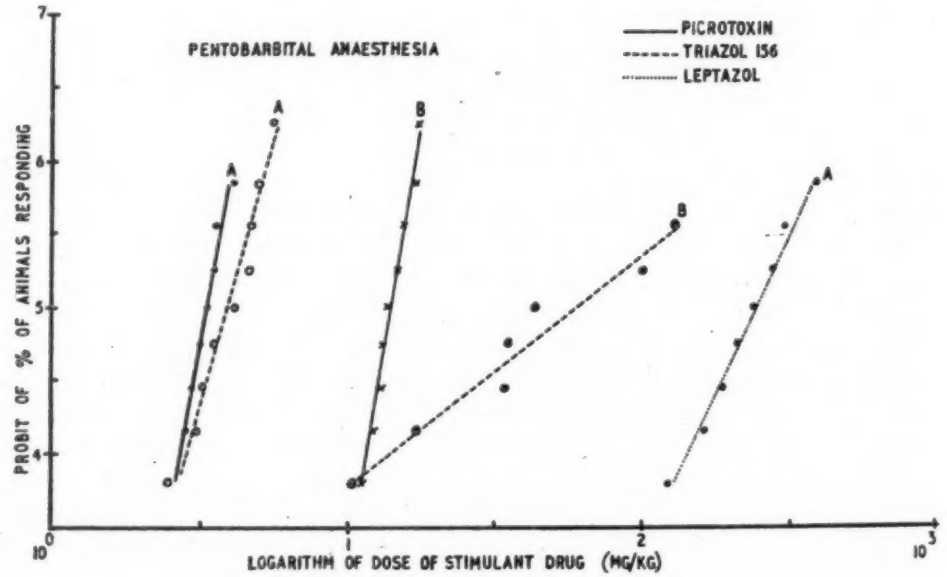


FIG. 2.

pentobarbital was used. This awakening effect was noted by Trevan, and can, in the mouse tests, give a false impression of the efficiency of this drug. Probably the best analeptic of the three tested is triazol 156, since it does not cause the muscular jerking shown by picrotoxin, although larger doses are required.

The method has been used to examine a series of synthetic compounds the results of which will be reported shortly. A compound can be examined in approximately three hours, and the method gives a result under conditions comparable with those of the projected therapeutic application. The method gives no numerical index of the duration of action of these drugs, but transient stimulation while injection is being performed has frequently been observed with new compounds. Whenever this test has indicated useful analeptic activity it has been our practice to use the method described by Das in order to assess the duration of action of the drug, given by various routes, but the method described here provides a valuable primary measure of stimulant activity.

Summary

A method of comparing the respiratory stimulant properties of analeptics is described. Guinea-pigs are anaesthetized by a continuous infusion of short- or long-acting barbiturates ; when respiration ceases for twenty seconds the analeptic is also infused. The doses of analeptic required both to restart respiration and to cause over-stimulation are measured.

A comparison of picrotoxin, leptazol, and triazol 156 by this method is described.

References

Behrens, B., Dinkler, G., and Woenckhaus, E. (1937). *Klin. Wschr.*, **16**, 944.
Chakravati, M. (1939). *J. Pharmacol.*, **67**, 153.
Das, S. C. (1939). *Quart. J. exp. Physiol.*, **29**, 355.
Trevan, J. W. (1939). *Proc. roy. Soc. Med.*, **32**, 391.

Brit. J. Pharmacol. (1947), **2**, 100.

THE BIOLOGICAL ESTIMATION OF SUBSTANCES USED IN TREATING CESTODE INFESTATIONS

BY

PAMELA HOLTON

From the Department of Pharmacology, Oxford

(Received December 12, 1946)

The first experimental observations of the action of anthelminthic drugs were made by von Schroeder (1884, 1885) on annelid worms. He was followed by Trendelenburg (1916), who used round worms, but mainly worked with annelids. Sollmann (1919) and Wasicky (1923) used annelid worms and fish. Hall (1921) was the first to carry out experiments on infested animals. He determined the percentage of parasitic worms which were expelled when dogs were given anthelminthics ; his main interest, however, was in nematodes. Rebello, Gomes da Costa, and Toscano Rico (1928 ; also Gomes da Costa, 1931) showed that cestodes and nematodes behave differently when treated with anthelminthics outside the body, as might be expected since they belong to different phyla of the animal kingdom. Gomes da Costa and his colleagues have developed a method for the observation of isolated segments of *Taenia saginata* and *Dipylidium caninum*. Their results have confirmed clinical findings and they have been able to recommend new drugs for trial (Gomes da Costa, 1930, 1932 ; Gomes da Costa and Hamet, 1935, 1937). They showed that the survival time of a segment of *Dipylidium caninum* in an oil bath bore an inverse relation to the concentration of filix mas, but made no further attempt to obtain a response graded to the dose (Ettisch and Gomes da Costa, 1937).

Culbertson (1940) worked with mice infested with *Hymenolepis fraterna* (called in this paper *Hymenolepis nana*) and found that doses of atabrine (mepacrine) greatly reduced the number of worms in the intestines of the mice. Again, however, no quantitative results were obtained.

The experiments described in this paper were undertaken to find a convenient method of determining anticestode activity as part of an attempt to isolate the active principles of certain crude drugs. The work has been done with one sample of extractum filicis B.P. Mice supplied to the laboratory were found to be naturally infested by two species of tapeworm, *Hymenolepis nana* and the much larger *Hymenolepis diminuta*. These two species cannot easily be distinguished by inspection of their eggs, so that infested mice may harbour either or

both species, but since *H. diminuta* is so much larger than *H. nana*, the results, which were obtained by weighing the worms, depend almost entirely on the infestation with *H. diminuta*. When fresh mice came to the laboratory, infestation was determined by microscopic examination of the faeces for eggs; as a rule about 30 per cent of the mice were infested. The mice in the faeces of which no eggs were detected were returned to the laboratory stock and were re-examined at intervals of about 3 weeks, when a further 10 per cent were found to be infested.

Details of Procedure

Examination of mice for infestation.—The mice were fed at least an hour before examination. A pellet of faeces was then taken from each mouse and a faecal smear was made on a microscope slide. Using low power focused on the bottom of the smear, it was fairly easy to spot the typical egg (see Fig. 1) containing a hexacanth embryo. When in doubt the high power was used.

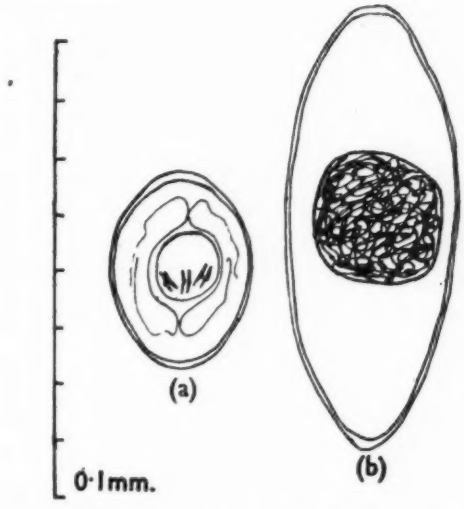


Fig. 1.—Helminth eggs found in mouse faeces. (a) *Hymenolepis* egg containing a hexacanth embryo. (b) Nematode egg. This is the only common egg likely to be mistaken for *Hymenolepis*.

When ext. filicis is used clinically, the patient is starved overnight. The next morning he is given 3–6 ml. of the extract, followed by a saline purge after two hours. This procedure was adopted with the infested mice, and the proportion of worms excreted was found. Preliminary experiments were carried out to find suitable doses of ext. filicis and of purgative.

Oral toxicity of ext. filicis.—The mouse dose, corresponding to a human dose of 6 ml., is 0.002 ml. An aqueous emulsion was made by shaking ext. filicis with sodium glycocholate (1 per cent) solution. This emulsion was not very stable, but was used for the first experiments. Later, when quantitative results were required, a standard emulsion of ext. filicis in ether and sodium glycocholate solution was prepared as described below.

Groups of mice were given by mouth doses of ext. filicis emulsion (1 and 10 per cent by volume). After 24 hours the dead mice were counted. The results are given in Table I.

TABLE I

ORAL TOXICITY OF EXTRACTUM FILICIS

Dose	Volume of emulsion administered	Result
50 mg.	0·5 ml. 10 per cent (v/v)	9 out of 15 mice died
20 mg.	0·2 ml. 10 „ „	0 „ 3 „ „
10 mg.	1·0 ml. 1 „ „	0 „ 3 „ „

Since the lethal dose was about 50 mg., one-fifth of this dose (10 mg. ext. filicis) was used as the highest therapeutic dose.

Choice of purgative.—It is usually taught that a saline purgative should be given after ext. filicis, since when castor oil is used the extract dissolves in the oil and is absorbed. The first experiments were therefore made with sodium sulphate as a purgative; this was found to produce diarrhoea when 0.1 g. (anhydrous) was given orally in 1 ml.; half this dose was not effective. Since the purgative effect was delayed in some experiments and might have been due to hypertonicity, 1 ml. of a solution of 6.5 g. $MgSO_4.7H_2O$ in 100 ml. water, which is isotonic, was used instead.

Examination of material.—Examination of faeces or gut contents was carried out in water on a black developing dish. The white fragments of worms were picked out with forceps and rinsed free of debris. At first all fragments were examined microscopically and the number of scolices of each species (*H. diminuta* and *H. nana*) was counted. It was then decided to weigh the worm fragments in a small Gooch crucible after they had been roughly dried with cotton wool and filter paper. An attempt to determine the dry weight after heating to 100°C. for 1 hour was unsuccessful because this weight was never more than a few milligrammes and consequently was too small to be an accurate measure of worm content. The wet weight was of the order of 100 mg. per mouse and was adopted as a convenient measure of the amount of worms. The results of an early experiment are given in Table II.

TABLE II

Material	Scolices			Weight of worm fragments	
Faeces	*H. nana*	*H. diminuta*	Total	Wet, mg.	Dry, mg.
10 Treated mice ..	5	18	23	711	44
10 Control mice ..	1	0	1	33	<1
Gut contents					
10 Treated mice ..	2	4	6	167	3
10 Control mice ..	122	13	135	787	38

Percentage of worms (wet weight) discharged by:

(1) Treated mice 81.

(2) Control mice 4.

In the early experiments the control mice often died, and the conclusion was drawn that the period without food was too long. Glucose injections were first tried as a means of providing calories without giving solid food, but these made no difference. Lumps of sugar were then put in the boxes, and this device was successful; the experiments were carried through without deaths in the control or treated mice provided that mice of not less than 18 g. weight were used.

The next difficulty was that mice receiving a high dose of ext. filicis often discharged a smaller percentage of tapeworms than mice receiving a lower dose. It was assumed that the worms were affected by the higher dose, but the mice were weakened and so did not excrete them in the five hours of the experiment. The solution of this problem was suggested by the fact that worms from the intestines of mice in control groups moved vigorously in water and could easily be separated from those which did not move and which were assumed to be killed or paralysed by the drug. To give the worms every chance of movement, the intestines were opened in warm saline. The " dead " worms and live worms from each group were picked out as described before and weighed separately. This division could always be made without hesitation when the worms were touched with forceps. As well as the " dead " worms in the small intestine, worms were sometimes found in the large intestine, including the caecum; these were obviously about to be excreted, because *Hymenolepis* does not inhabit this part of the gut, and they were included in the total of affected worms.

This revised technique was used in eleven experiments. In each experiment groups of five infested mice (each mouse weighing over 18 g.), which had been fed on lumps of sugar from 6 p.m. the previous day, were given doses of freshly prepared filicis emulsion, followed by magnesium sulphate as a purgative after two hours. Three hours later the faeces of each group were examined as already described. Each mouse was then killed and its intestine was removed. The small intestine was put into warm (38°C.) saline in a black dish and slit open longitudinally with scissors. The mucosa was scraped with a microscope slide and the intestinal contents were examined as described above. The large intestine was treated in the same way. The three categories of worms from the intestines of each group were weighed separately. The weight of worms affected by the drug was expressed as a percentage of the total weight of worms in each group. The method is illustrated by the following experiment:

Exp. 17. Preparation of ext. filicis emulsion.—After being stirred the ext. filicis (230 mg., or four drops) was weighed in a 50-ml. conical flask and dissolved in 1 ml. of ether; distilled water, containing about 1 per cent sodium glycocholate, was added so that 1 ml. of the resulting emulsion contained exactly 10 mg. ext. filicis. The emulsion was shaken before use and diluted when necessary with a solution of glycocholate and ether in the same proportions as above so that a volume of 1 ml. was administered to each mouse.

The ext. filicis emulsion and the purgative were administered with a blunted hypodermic needle, used as a stomach tube, and a 1-ml. syringe.

Timetable of experiment

9.40 a.m. Group 1. 5 mice given by mouth 10 mg. ext. filicis.
Group 2. „ „ „ 5 mg. ext. filicis.
Group 3. „ „ „ 2.5 mg. ext. filicis.
Group 4. „ „ „ 1.25 mg. ext. filicis.

Each group was put into a wooden box with a metal floor and supplied with 4 lumps of sugar and water.

11.40 a.m. Each mouse was given by mouth 1 ml. warm 6.5 per cent (w/v) magnesium sulphate solution. The groups were replaced in their boxes.

2.40 p.m. Examination of faeces scraped from the floor of each box.
Examination of intestines.

The results are given in Table III.

TABLE III

Figures are weights of worms (mg.)

	Group 1	Group 2	Group 3	Group 4
Dose of ext. filicis:	10 mg.	5 mg.	2·5 mg.	1·25 mg.
Worms in faeces (*a*)	53	235	286	121
Live worms in small intestine (*b*)	0	75	290	771
" Dead " worms in small intestine (*c*) ..	0	41	0	0
Worms in large intestine (*d*)	65	130	115	0
Total worms ($a + b + c + d$)	118	481	691	892
Total worms affected by drug ($a + c + d$)	118	406	401	121
Percentage wet weight of worms affected by drug	100	85	58	13

The results of the eleven experiments are given in Table IV. A total of 220 mice were used ; 55 for each dose of ext. filicis.

TABLE IV

PERCENTAGE WET WEIGHT OF TAPEWORMS AFFECTED BY DOSES OF EXT. FILICIS GIVEN TO GROUPS OF 5 MICE

	Group 1	Group 2	Group 3	Group 4
Dose of ext. filicis:	10 mg.	5 mg.	2·5 mg.	1·25 mg.
Experiment 14 ..	100	55	48	0
15 ..	91	18	9	0
16 ..	100	88	38	6
17 ..	100	85	58	13
18 ..	100	59	90	0
19 ..	67	85	0	0
20 ..	100	33	67	44
21 ..	96	100	8	0
22 ..	100	45	58	52
23 ..	86	92	40	28
24 ..	85	100	92	14
Mean ..	92	69	46	14

It will be noticed that in any single experiment (e.g., expts. 18, 19) the response is not necessarily graded to the dose, but if the results of any three consecutive experiments are taken together the mean percentage wet weights of tapeworms affected are graded to the doses. This shows that at least fifteen mice must be used for every dose of anticestode drug. The results are shown graphically in Figs. 2 and 3. In Fig. 2 the mean percentage effect was plotted against

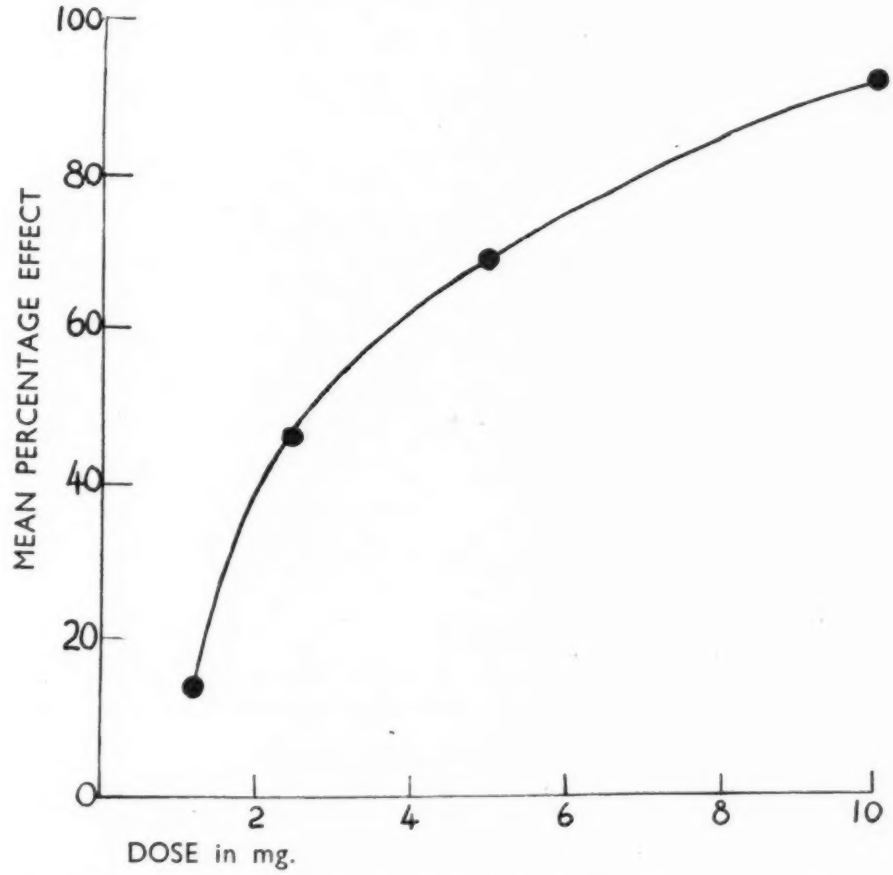


FIG. 2.—Results of 11 experiments using a total of 55 mice for each dose of ext. filicis. Ordinates: Mean percentage wet weight of tapeworms affected. Abscissae: Dose (mg.) given to each mouse.

each dose and a curve was drawn relating dose to effect. In Fig. 3 the expected probits of the mean percentage effect, calculated as described by Bliss (1938), were plotted against the logarithms of the doses. The slope of the line, $b=2.28$. The probability of the line, P, is approximately 0.02.

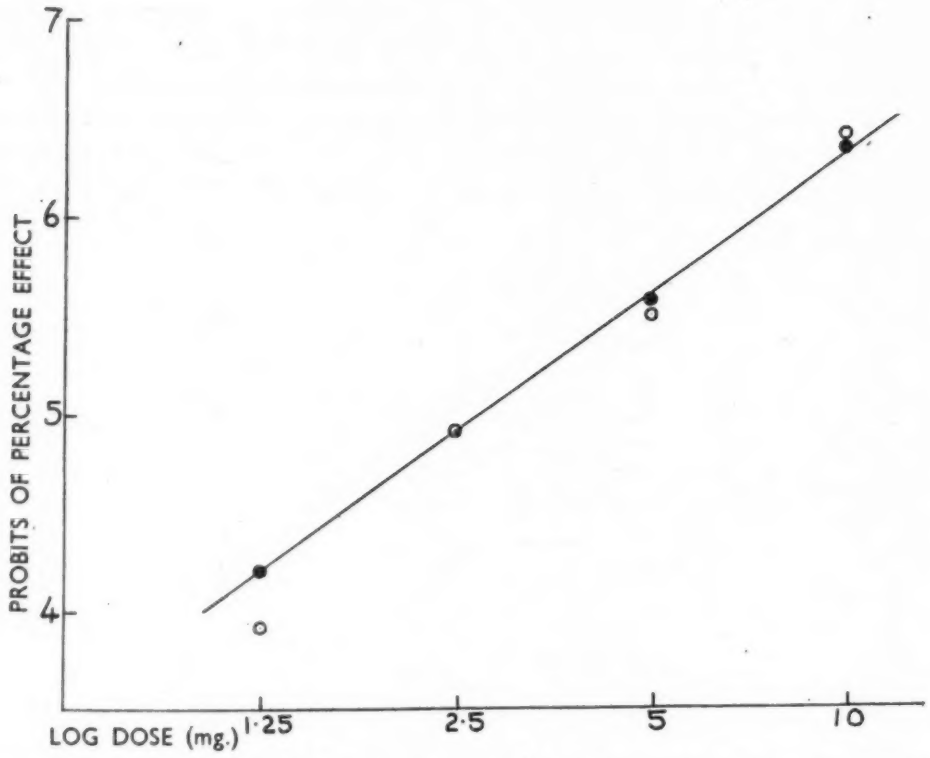


FIG. 3.—Same results as in Fig. 2. Ordinates: Probits of mean percentage wet weight of tapeworms affected. Abscissae: Log dose (mg.) given to each mouse. White circles: Empirical probits. Black circles: Expected probits.

SUMMARY

A method for the standardization of anticestode drugs is described. Mice infested with *Hymenolepis diminuta* were used as experimental animals. The mice were fed on lumps of sugar only for 15 hours. Then each mouse was given a dose of ext. filicis in 5 per cent ether emulsion using a stomach tube. Two hours later each mouse was given a dose of magnesium sulphate solution as a purgative. After another three hours the faeces were examined for *Hymenolepis* fragments, which were collected and weighed. The mice were killed and worms from each group were divided into three categories, which were weighed separately.

1. Worms from the large intestine (not normally inhabited by *Hymenolepis*).
2. " Dead " worms from the small intestine.
3. Live worms from the small intestine.

The weight of the affected worms (categories 1 and 2 and worms in the faeces) was expressed as a percentage of the total weight of worms. Using four different

doses of ext. filicis in a total of 220 mice (55 per dose), it was found that the probits of the percentage wet weight of worms affected increased in linear relation to the logarithm of the dose.

This work was carried out as a result of an inquiry from the Colonial Products Research Council whether substances could be tested for activity against tapeworms. The work has been done at the suggestion and under the supervision of Prof. J. H. Burn. My thanks are due to Prof. R. T. Leiper, who very kindly arranged for me to be shown the details of *Hymenolepis* infestation in mice.

REFERENCES

Bliss, C. I. (1938). *Quart. J. Pharm. Pharmacol.*, **11**, 192.
Culbertson, J. T. (1940). *J. Pharmacol.*, **70**, 309.
Ettisch, G., and Gomes da Costa, S. F. (1937). *C. R. Soc. Biol., Paris*, **125**, 560.
Gomes da Costa, S. F. (1930). *C. R. Soc. Biol., Paris*, **103**, 342, 345.
Gomes da Costa, S. F. (1931). *Arch. int. Pharmacodyn.*, **41**, 443.
Gomes da Costa, S. F. (1932). *C. R. Soc. Biol., Paris*, **110**, 1054.
Gomes da Costa, S. F., and Hamet, R. (1935). *Arch. int. Pharmacodyn.*, **50**, 237.
Gomes da Costa, S. F., and Hamet, R. (1937). *Arch. int. Pharmacodyn.*, **56**, 314.
Hall, M. C. (1921). *J. agric. Res.*, **21**, 157.
Rebello, S., Gomes da Costa, S. F., and Toscano Rico, J. (1928). *Helmintiases e Anti-helminticos*, Lisbon.
von Schroeder, W. (1884). *Arch. exp. Path. Pharmak.*, **18**, 381.
von Schroeder, W. (1885). *Arch. exp. Path. Pharmak.*, **19**, 291.
Sollmann, T. (1919). *J. Pharmacol.*, **12**, 129.
Trendelenburg, P. (1916). *Arch. exp. Path. Pharmak.*, **79**, 190.
Wasicky, R. (1923). *Arch. exp. Path. Pharmak.*, **97**, 454.

Brit. J. Pharmacol. (1947), **2**, 108.

THE AFFINITY OF ATROPINE-LIKE ESTERS FOR ESTERASES

BY

H. BLASCHKO, T. C. CHOU, AND ISABELLE WAJDA

From the Department of Pharmacology, Oxford

(Received December 27, 1946)

In a recent paper from this department observations on a number of esters with atropine-like actions were reported (Ing, Dawes, and Wajda, 1945). The substances studied included the choline esters of benzilic, tropic, and atrolactinic acid, as well as esters of benzilic acid in which choline was replaced by other basic alcohols. In the experiments to be described we have investigated the behaviour of these substances towards esterases, and in particular their affinity for the cholinesterases, by testing whether or not they inhibited the hydrolysis of esters known to be substrates of these enzymes. In addition, we have examined two other esters of interest to the pharmacologist: (a) cocaine, and (b) the diethylaminoethyl ester of *cyclo*hexyl-phenylacetic acid (trasentin 6H).

Mammalian tissues contain a number of different enzymes which will hydrolyse acetylcholine and other choline esters (Alles and Hawes, 1940; Richter and Croft, 1942; Mendel and Rudney, 1943; Zeller and Bissegger, 1943; Nachmansohn and Rothenberg, 1945). Mendel and Rudney have shown that two different types of cholinesterase can be characterized by using acetylcholine and benzoylcholine as substrates; they distinguish the "true" cholinesterase from the "pseudo"-cholinesterase. The former will hydrolyse acetylcholine only, the latter both acetylcholine and benzoylcholine. We have found Mendel's nomenclature useful in describing our results and it has therefore been employed in this paper. In addition to these two types of enzymes, preparations have been described which will hydrolyse benzoylcholine, but not acetylcholine. The guinea-pig liver (Sawyer, 1945) and the ox kidney (Gunter, 1946) each contain an enzyme of this kind.

Rabbit serum contains an enzyme, tropinesterase, which hydrolyses atropine, and it was naturally of interest to study the behaviour of the synthetic atropine substitutes towards this enzyme. Tropinesterase does not occur in all rabbits; its presence is genetically determined (Sawin and Glick, 1943).

A few observations were made on an enzyme which hydrolyses tropacocaine in horse serum. The occurrence of this enzyme, first reported by Glick and Glaubach (1941), was confirmed.

The tissues and substrates used are listed in Table I and the substances tested in Table II.

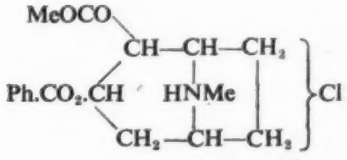
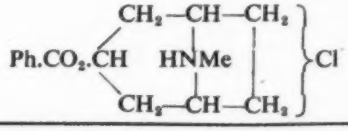
TABLE I

LIST OF TISSUES AND SUBSTRATES USED

Species	Tissue	Amount of tissue used	Substrate	Concentration
Dog ..	Caudate nucleus	5–10 mg.	Acetylcholine bromide	$6 \times 10^{-3}M$
Horse ..	Serum	0.2 ml.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Guinea-pig	Liver	12.5–25 mg.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Ox	Kidney	1.0–1.3 g.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Rabbit* ..	Serum*	0.15 ml.	Atropine sulphate	1 g./100 ml.
Horse ..	Serum	0.3 ml.	Tropacocaine hydrochloride	$10^{-2}M$

* tropinesterase-positive.

TABLE II

	Substance	Structure
Quaternary bases	Lachesine (E3) or benzilyloxyethyldimethylethylammonium chloride	$Ph_2.C(OH).CO_2.CH_2.CH_2.NMe_2Et\}Cl$
	Benzilylcholine chloride (C1)	$Ph_2.C(OH).CO_2.CH_2.CH_2.NMe_3\}Cl$
	Tropylcholine chloride	$Ph.CH(CH_2OH).CO_2.CH_2.CH_2.NMe_3\}Cl$
	Atrolactylcholine chloride	$Ph.CMe(OH).CO_2.CH_2.CH_2.NMe_3\}Cl$
Tertiary bases	Dimethylaminoethyl benzilate hydrochloride (C4)	$Ph_2.C(OH).CO_2.CH_2.CH_2.NMe_2H\}Cl$
	Diethylaminoethyl benzilate hydrochloride (E1)	$Ph_2.C(OH).CO_2.CH_2.CH_2.NEt_2H\}Cl$
	Trasentin 6H or diethylaminoethyl phenyl*cyclo*hexylacetate hydrochloride	$Ph(C_6H_{11})C(OH).CO_2.CH_2.CH_2.NEt_2H\}Cl$
	Cocaine hydrochloride	
	Tropacocaine hydrochloride	

Serial numbers in parentheses are taken from Ing *et al.* (1945).

Materials and Methods

Enzyme extracts were prepared by grinding the appropriate organs with sand; Krebs's Ringer-bicarbonate was used as the medium. No sand was added in preparing extracts of caudate nucleus, a suspension of the thoroughly ground tissue being used. No attempt was made to purify the enzyme preparations used, and it should be borne in mind that impurities may have influenced the affinities of the substances tested in this and the following paper.

Non-enzymic hydrolysis during the experiments occurred to a very small extent with the quaternary ammonium bases, but benzilic esters with a tertiary basic group showed a larger spontaneous hydrolysis which gave rise to a small blank. Trasentin 6H gave a slight precipitate with the Ringer solution, and its actual concentration is therefore somewhat uncertain.

The hydrolysis was followed manometrically; the experiments were carried out at a temperature of 38° with a gas mixture containing 95 per cent N_2 and 5 per cent CO_2.

Results

(1) *Hydrolysis of acetylcholine by dogs' caudate nucleus* (Table III).—Preparations of the caudate nucleus contain a powerful cholinesterase (Nachmansohn, 1937). This enzyme is a true cholinesterase (Mendel and Rudney, 1943).

Not one of the esters examined was hydrolysed by this preparation. We also tested their affinity for the enzyme by measuring their effect on the rate of hydrolysis of acetylcholine. They had very little action. The strongest effect was found with cocaine, which, in a concentration equimolecular to that of acetylcholine, caused an inhibition of 50 per cent. With trasentin 6H the inhibition was less, and none of the benzilic esters had any inhibitory activity in the concentration used.

TABLE III

EXTRACT OF THE CAUDATE NUCLEUS OF THE DOG

Hydrolysis of acetylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	0
Benzilylcholine (C1)	$6 \times 10^{-3}M$	0
Tropylcholine	$6 \times 10^{-3}M$	0
C4	$6 \times 10^{-3}M$	0
E1	$6 \times 10^{-3}M$	0*
Trasentin 6H	$6 \times 10^{-3}M$	15
Cocaine	$6 \times 10^{-3}M$	50

* This experiment was carried out with an extract of rabbit's basal ganglia.

(2) *Hydrolysis of benzoylcholine by horse serum* (Table IV).—Horse serum contains an active pseudo-cholinesterase, and in order to exclude the action of true cholinesterase we used benzoylcholine as substrate. The same sample of serum was used in all these experiments.

Horse serum hydrolysed atrolactylcholine; these observations are described separately in section (6). None of the other esters was hydrolysed, but they all had an affinity for the horse serum esterase: all the substances tested inhibited the enzyme, and with cocaine and trasentin the percentage inhibitions were higher than for true cholinesterase.

TABLE IV

HORSE SERUM

Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	71
Benzilylcholine (C1)	$6 \times 10^{-3}M$	33
Tropylcholine	$6 \times 10^{-3}M$	43
Trasentin 6H	$6 \times 10^{-3}M$	81
Cocaine	$6 \times 10^{-3}M$	85

(3) *Hydrolysis of benzoylcholine in guinea-pig liver* (Table V).—This preparation contains no pseudo-cholinesterase (Blaschko, Chou, and Wajda, 1947); the hydrolysis of benzoylcholine is solely due to the benzoylcholinesterase described by Sawyer (1945). Whether or not benzoylcholine is the normal substrate of the enzyme in the living animal is unknown.

All the esters examined acted as inhibitors of the enzyme (see Table V) and the percentage inhibitions were higher than for either true or pseudo-cholinesterase. Not one of the esters was hydrolysed by the enzyme, with the possible

TABLE V

GUINEA-PIG'S LIVER EXTRACT

Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	94
" "	$6 \times 10^{-4}M$	91
" "	$6 \times 10^{-5}M$	66
C4	$6 \times 10^{-3}M$	100
E1	$6 \times 10^{-3}M$	100
Trasentin 6H	$1.2 \times 10^{-3}M$	97
" "	$1.2 \times 10^{-4}M$	88
" "	$1.2 \times 10^{-5}M$	80
" "	$1.2 \times 10^{-6}M$	45
Cocaine	$6 \times 10^{-3}M$	100
"	$6 \times 10^{-4}M$	92
"	$6 \times 10^{-5}M$	44

exception of trasentin 6H, where with the lowest concentration used ($1.2 \times 10^{-6}M$) the percentage inhibitions during the first 15 min. period were consistently higher than in the second.

(4) *Hydrolysis of benzoylcholine in ox kidney* (Table VI).—The benzoylcholinesterase of ox kidney described by Gunter (1946) has not yet been fully analysed. The enzymic activity of the tissue extracts is very much less than that of the extracts of guinea-pig liver, and it was therefore necessary to use much larger amounts of tissue (see Table I).

Our results with this preparation showed a striking difference from those with the guinea-pig liver extract: the esters examined had little or no effect on the rate of hydrolysis of benzoylcholine.

TABLE VI

OX KIDNEY EXTRACT

Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	12
Benzilylcholine (C1)	$6 \times 10^{-3}M$	19
C4	$6 \times 10^{-3}M$	0 (approx.)
E1	$6 \times 10^{-3}M$	29 (approx.)
Trasentin 6H	$10^{-3}M$	0
Cocaine	$10^{-3}M$	14

(5) *Hydrolysis of atropine in rabbit serum* (Table VII).—All experiments were carried out with the sera of two animals which were found to contain tropinesterase; they were among a group of about a dozen animals tested for the presence of the enzyme. Not one of the substances included in Table VII showed any enzymic hydrolysis. Cocaine was not included, as it was found to be hydrolysed enzymically; we confirmed Glick and Glaubach's (1941) observation that this hydrolysis also occurs in the serum of animals without tropinesterase.

The benzilic esters had no inhibitory action on tropinesterase (see Table VII); of all the substances examined only trasentin 6H had a slight inhibitory effect.

TABLE VII

RABBIT'S SERUM

Hydrolysis of atropine sulphate (1 g./100 ml.) by tropinesterase

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$10^{-2}M$	0
Benzilylcholine (C1)	$10^{-2}M$	0
Atrolactylcholine	$6 \times 10^{-3}M$	0
Trasentin 6H	$10^{-3}M$	60

(6) *Enzymic hydrolysis of atrolactylcholine in horse serum.*—None of the synthetic esters examined showed any detectable enzymic hydrolysis with the exception of the choline ester of atrolactinic acid in horse serum. Only small amounts of the substance were at our disposal, but the following facts were established: the ester showed an appreciable hydrolysis in Ringer solution, but the rate of liberation of carbon dioxide was consistently higher in the presence of horse serum. That this hydrolysis in excess of the blanks was due to an enzyme is supported by the following observations:

(i) the hydrolysis increased with increasing amounts of serum; in one experiment the additional amount of CO_2 liberated in 15 min. by 0.2 ml. of horse serum was 9.5 μl., in another experiment with 0.4 ml. of serum it was 19 μl. (the figures for spontaneous hydrolysis in the blanks were 7.5 and 9.5 μl. respectively);

(ii) the additional hydrolysis was abolished by boiling the serum; and

(iii) it was reduced in the presence of $1.8 \times 10^{-5} M$ eserine.

In addition the following facts were established: there was no enzymic hydrolysis of atrolactylcholine in rabbit serum (0.15 ml. per flask) and in dog's caudate nucleus extract (equivalent of 40 mg. fresh weight of tissue per flask).

These results strongly suggest that atrolactylcholine is a substrate of pseudo-cholinesterase. Mendel *et al.* (1943) have shown that the serum of the horse has about 5 times as much pseudo-cholinesterase activity as that of the rabbit. Moreover, the eserine inhibition of the hydrolysis of both benzoyl- and atrolactyl-cholines were of the same order: in the presence of $1.8 \times 10^{-5} M$ eserine the hydrolysis of $6 \times 10^{-3} M$ benzoylcholine was reduced, the CO_2 output falling from 102 to 38.5 μl. in 15 min., and for atrolactylcholine, used in the same concentration, the corresponding figures were 15 and 9 μl. CO_2 respectively. It was also shown that atrolactylcholine had an affinity to pseudo-cholinesterase, as the hydrolysis of benzoylcholine was reduced in the presence of the atrolactinic ester. The amounts of carbon dioxide liberated by 0.2 ml. of horse serum in 15 min. were:

with $6 \times 10^{-3} M$ benzoylcholine—117 μl.
with $6 \times 10^{-3} M$ atrolactylcholine—9.5 μl.
with both esters—58.5 μl.

(7) *Hydrolysis of tropacocaine in horse serum* (Table VIII).—None of the substances examined had any affinity for the enzyme in the concentrations used

TABLE VIII

HORSE SERUM

Hydrolysis of tropacocaine ($10^{-2} M$)

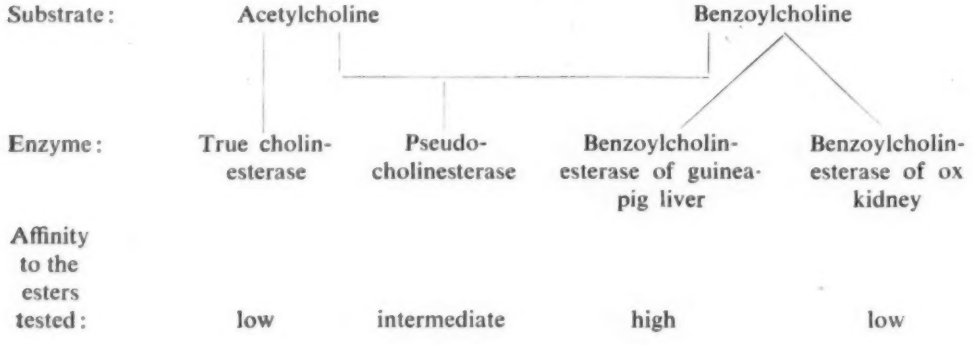
Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$10^{-3} M$	0
Benzilylcholine (C1)	$10^{-3} M$	0
Cocaine	$10^{-3} M$	0
Atropine	$10^{-3} M$	0

in our experiments. The enzyme responsible for the hydrolysis of tropacocaine must therefore be distinct from pseudo-cholinesterase, as this enzyme is inhibited by some of the substances listed in Table VIII, e.g., cocaine, lachesine, and benzilylcholine.

Discussion

Our observations provide an example of the usefulness of inhibitors for distinguishing related enzymes. All the enzymes studied were esterases and yet great differences in their affinities to the esters tested were found. The degree of inhibition is determined not by the substrate used, which may or may not be common to two enzymes, but by the specific affinities of the enzyme itself. With acetylcholine as substrate this was first clearly demonstrated in experiments by Zeller (1942), who found that the human serum cholinesterase was much more sensitive to *iso*propyl-antipyrin than the cholinesterase of the central nervous system.

Our results can be summarized in the following scheme:



All the substances tested had little affinity to true cholinesterase, more to pseudo-cholinesterase, and the highest affinity to the benzoylcholinesterase of guinea-pig liver; the benzoylcholinesterase of ox kidney was little or not at all inhibited. The activity of the ox kidney was very low and the amount of tissue used had therefore to be very much greater than with any of the other preparations (Table I). The possibility that the small inhibitor action in this case is due to a reaction of the esters with some other constituents in the ox kidney cannot therefore be excluded, but it seems more likely that the benzoylcholinesterase of ox kidney differs from the corresponding enzyme in the liver of the guinea-pig.

We have not found any enzymic hydrolysis of lachesine or any of the other esters of benzilic acid tested as atropine substitutes by Ing *et al.* (1945). It is interesting that these substances which must have a great affinity for the tissue receptors on which atropine acts have little or no affinity for the enzyme tropinesterase.

Our experiments on atrolactylcholine increase the number of choline esters known to be hydrolysed in animal tissue ; it seems interesting that the chemically closely related esters of tropic and benzilic acids were not hydrolysed.

Cocaine, an ester of benzoic acid, has a much higher affinity for pseudo-cholinesterase, which hydrolyses the benzoic ester of choline, than for the true esterase which does not (see also Nachmansohn and Schneemann, 1945). Cocaine also inhibits the benzoylcholinesterase of guinea-pig's liver, but neither the benzoylcholinesterase of ox kidney nor the tropacocainesterase of horse serum was inhibited by cocaine ; this shows that affinity for cocaine is not a general property of esterases which hydrolyse esters of benzoic acid.

Summary

1. The action of cocaine, benzilylcholine, lachesine, and a number of related atropine-like esters on cholinesterases and on tropinesterase has been studied.

2. These substances were found to have little affinity to the " true " cholinesterase of brain tissue, more affinity to the " pseudo "-cholinesterase of horse serum, and a high affinity to the benzoylcholinesterase of guinea-pig's liver. They had little or no effect on the hydrolysis of benzoylcholine by ox kidney extracts.

3. They had little or no affinity to the tropinesterase of rabbit's serum.

4. Evidence is given which suggests that atrolactylcholine is hydrolysed by pseudo-cholinesterase of horse serum.

5. The tropacocainesterase of horse serum was not inhibited by these substances, which shows that it is not identical with pseudo-cholinesterase.

We are grateful to Dr. H. R. Ing for the benzoylcholine and the esters prepared by Ford-Moore and Ing (1947), and to Dr. R. Wien for the tropacocaine used in these experiments.

Two of us (H. B. and I. W.) are indebted to the Medical Research Council for personal grants held while this investigation was carried out.

References

Alles, G. A., and Hawes, R. C. (1940). *J. biol. Chem.*, **133**, 375.
Blaschko, H., Chou, T. C., and Wajda, I. (1947). *Brit J. Pharmacol.*, **2**, 116.
Ford-Moore, A. H., and Ing, H. R. (1947). *J. chem. Soc.*, 55.
Glick, D., and Glaubach, S. (1941). *J. gen. Physiol.*, **25**, 197.
Gunter, J. M. (1946). *Nature, Lond.*, **157**, 369.
Ing, H. R., Dawes, G. S., and Wajda, I. (1945). *J. Pharmacol.*, **85**, 85.
Mendel, B., Mundell, D. B., and Rudney, H. (1943). *Biochem. J.*, **37**, 473.
Mendel, B., and Rudney, H. (1943). *Biochem. J.*, **37**, 59.
Nachmansohn, D. (1937). *C. r. Soc. Biol., Paris*, **126**, 783.
Nachmansohn, D., and Rothenberg, M. A. (1945). *J. biol. Chem.*, **158**, 653.
Nachmansohn, D., and Schneemann, H. (1945). *J. biol. Chem.*, **159**, 239.
Richter, D., and Croft, P. G. (1942). *Biochem. J.*, **36**, 746.
Sawin, P. B., and Glick, D. (1943). *Proc. Nat. Acad. Sci.*, **29**, 55.
Sawyer, C. H. (1945). *Science*, **101**, 385.
Zeller, E. A. (1942). *Helv. chim. Acta*, **25**, 1099.
Zeller, E. A., and Bissegger, A. (1943). *Helv. chim. Acta*, **26**, 1619.

Brit. J. Pharmacol. (1947), **2**, 116.

THE INHIBITION OF ESTERASES BY PALUDRINE

BY

H. BLASCHKO, T. C. CHOU, AND ISABELLE WAJDA

From the Department of Pharmacology, Oxford

(Received December 27, 1946)

Many observations on the action of antimalarial drugs on ester-splitting enzymes have been recorded. Rona and Reinicke (1921) have shown that quinine inhibits the esterase which hydrolyses tributyrin in human serum; this enzyme is also inhibited by many other antimalarials, e.g., pamaquin, optochin, and mepacrine (Fulton, 1936). Both quinine and mepacrine act on cholinesterases: quinine has been shown to inhibit the enzyme now generally known as pseudo-cholinesterase (Nachmansohn and Schneemann, 1945); mepacrine inhibits the hydrolysis of acetylcholine by both pseudo- and true cholinesterases (Waelsch and Nachmansohn, 1943).

It therefore seemed interesting to examine the effect of paludrine on esterases. Paludrine differs greatly from the other antimalarials in its chemical constitution and is characterized by a low toxicity. The latter fact made it unlikely that it would strongly inhibit an enzyme so vital as the "true" cholinesterase of the central nervous system and muscles, but it is known that there are other enzymes which will hydrolyse choline esters. We have therefore examined the action of paludrine on all the known cholinesterases and on a number of related enzymes.

MATERIAL AND METHODS

Most of these have been described in the preceding paper (Blaschko, Chou, and Wajda, 1947). In addition we have examined the hydrolysis of acetylcholine by plasma and red-cell haemolysate. The hydrolysis of tributyrin was also studied in human serum and in cat's liver extracts. The cat's liver used for these experiments was washed free from blood *in situ* by perfusion with Locke's fluid.

EXPERIMENTS

1. *Cholinesterases* (Table I)

Two preparations, representative of true cholinesterase, were examined: an extract from the dog's caudate nucleus and a haemolysate of human red cells. Both were inhibited slightly by paludrine. The rate of hydrolysis of acetylcholine ($6\times10^{-3}M$) by the brain extracts was reduced by about 22 per cent in the presence of $10^{-3}M$ paludrine. For the human haemolysate the inhibition was 49 per cent with $10^{-3}M$ paludrine; with $10^{-4}M$ paludrine there was no inhibition.

In the plasma (or serum) of many mammals the enzyme acting on acetylcholine is pseudo-cholinesterase. This enzyme which hydrolyses benzoylcholine as well as acetylcholine was more strongly inhibited by paludrine. In one experiment with human plasma and acetylcholine as substrate the percentage inhibitions were 92 per cent with $10^{-3}M$, and 60 per cent with $10^{-4}M$ paludrine. Similar inhibitions were observed with cat's plasma and horse serum as sources of the enzyme.

TABLE I

ACTION OF PALUDRINE ON CHOLINESTERASES

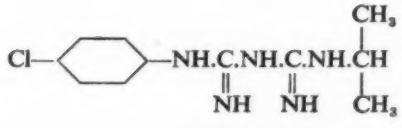
Substrate concentration $6 \times 10^{-3}M$

Species and tissue	Amount of tissue	Substrate used	Percentage inhibition by paludrine		
			$10^{-3}M$	$10^{-4}M$	$10^{-5}M$
Dog's caudate nucleus	4 mg.	acetylcholine	22	—	—
Human red-cell haemolysate	0.1 ml.	,,	49	0	—
Human plasma	0.05 ml.	,,	92	60	—
Horse serum	0.3 ml.	benzoylcholine	67	35	—
Cat plasma	0.005 ml.	acetylcholine	73	—	—
Guinea-pig liver	12.5 mg.	benzoylcholine	94	76	26
Ox kidney	1,000 mg.	,,	0	—	—

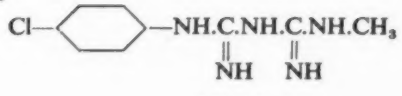
Benzoylcholinesterase hydrolyses benzoylcholine, but not acetylcholine; the enzyme has been found in the liver of the guinea-pig (Sawyer, 1945) and in ox kidney (Gunter, 1946). It is known that the latter organ does not contain pseudo-cholinesterase, and our experiments show that the same is true for the guinea-pig's liver. This was established by comparing the rates of hydrolysis of benzoylcholine with and without eserine, which is a strong inhibitor of pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943). We find that the rate of hydrolysis of benzoylcholine is the same in the presence or absence of 2×10^{-4} eserine, a concentration which completely inhibits pseudo-cholinesterase.

The affinities of paludrine for the two preparations of benzoylcholinesterase are very different. Whereas the ox kidney preparation was not inhibited by $10^{-3}M$ paludrine, this concentration rendered the enzyme from guinea-pig's liver almost completely inactive.

It seemed interesting to compare the effect of paludrine on benzoylcholinesterase with that of a related compound which has no antimalarial action, at least on *P. Gallinaceum* in chicks (Curd and Rose, 1946). Such a compound is the N_5-methyl homologue of paludrine, the substance M5093. In this substance the *iso*propyl group of paludrine is replaced by a methyl group.



Paludrine



M5093

The anti-benzoylcholinesterase activity of M5093 is very much less than that of paludrine. In one experiment we compared the inhibition with paludrine and M5093, both in $10^{-3}M$ concentrations, on the benzoylcholinesterase of guinea-pig's liver. The inhibitions were:

with paludrine 91 per cent; with M5093 42 per cent.

In another experiment the inhibitions were:

with $4 \times 10^{-5} M$ paludrine 49 per cent; with 10^{-3} M5093 41 per cent.

The latter experiment shows that the N-methyl homologue was less active as an inhibitor when present in a concentration 25 times that of paludrine.

M5093 had no inhibitory action on the true cholinesterase from dog's brain in a molar concentration of 10^{-3}.

2. *Tributyrinesterases* (Table II)

Rona and his collaborators have shown that the effect of quinine on the enzymic hydrolysis of tributyrin differs for preparations obtained from different organs: the tributyrinesterase of serum is strongly inhibited by quinine (Rona and Reinicke, 1921), but the esterase from liver is resistant to quinine (Rona and Pavlovic, 1922).

Both paludrine and mepacrine behave similarly to quinine. In the presence of $10^{-3} M$ paludrine the tributyrinesterase of human serum was almost completely inhibited and with $10^{-4} M$ paludrine the inhibition was 82 per cent. With $10^{-3} M$ mepacrine the inhibition was 92 per cent. On the other hand, with the preparation from cat's liver, the rate of hydrolysis of tributyrin was not affected in the presence of $10^{-3} M$ paludrine and of $10^{-3} M$ mepacrine.

It is interesting that the three antimalarial substances, so very different in their chemical constitution, should resemble each other so closely in their affinities for this enzyme, but it must be pointed out that the compound M5093, which is without antimalarial activity, behaves in a similar fashion. In a concentration of $10^{-3} M$ it was without inhibitory action on the enzyme from cat's liver, but with human serum the inhibition was 72 per cent.

TABLE II

INHIBITION OF TRIBUTYRINESTERASES

Substrate concentration 0.015 *M*

Species	Tissue	Amount of tissue	Inhibitor	Percentage inhibition at different concentrations		
				$10^{-3} M$	$10^{-4} M$	$10^{-5} M$
Man	Serum	0.2 ml.	paludrine	94	81	24
Cat	Liver	0.667 mg.	,,	2	—	—
Rabbit	Pancreas	20 mg.	,,	0	—	—
Rabbit	Kidney	200 mg.	,,	0	—	—
Man	Serum	0.2 ml.	mepacrine	92	87	60
Cat	Liver	0.667 mg.	,,	0	0	0
Man	Serum	0.2 ml.	M5093	73	36	8
Cat	Liver	0.667 mg.	,,	0	—	—

The hydrolysis of tributyrin in rabbit's kidney and pancreas was not inhibited by $10^{-3} M$ paludrine.

3. *Other esterases* (Table III)

Paludrine had no effect on the hydrolysis of methyl butyrate by rabbit's pancreas. It had a slight inhibitory action on the tropinesterase of rabbit's serum: with $10^{-3} M$ paludrine the inhibition was 48 per cent. We have also examined the tropacocainesterase of horse serum described by Glick and Glaubach (1941); this enzyme was not inhibited by paludrine.

TABLE III

ACTION OF PALUDRINE ON OTHER ESTERASES

Paludrine concentration $10^{-3}M$

Species and tissue	Amount of tissue used	Substrate used	Percentage inhibition
Rabbit pancreas	20 mg.	0.015 *M* methylbutyrate	0
Rabbit serum*	0.15 ml.	1 g./100 ml. atropine sulphate	48
Horse serum	0.3 ml.	0.01 *M* tropacocaine hydrochloride	0

* The serum was from a rabbit which contained tropinesterase.

DISCUSSION

The mechanism of antimalarial activity is a matter of speculation, but it seems likely that the therapeutic action is due to the drugs interfering with metabolic reactions of vital importance to the parasites; such interference is most easily thought of as an inhibition of enzymic reactions. Plasmodial enzymes have not yet been available for study, but it has long been known that some antimalarials will inhibit certain mammalian enzymes. Paludrine shares this property with such well-known antimalarials as quinine and mepacrine, but also displays some differences; it has, for example, a very low affinity for one of the physiologically most important enzymes, namely the true cholinesterase. Its most powerful inhibitory action in our experiments was on the benzoylcholinesterase of guinea-pig's liver, an enzyme so far characterized by a substrate which has not yet been shown to occur in animal tissues.

There is no general parallelism between antimalarial and anti-esterase properties, but the fact that so many antimalarials are at the same time inhibitors of esterases makes the suggestion that these substances act by inhibiting an esterase of importance in the life cycle of the malaria parasite worthy of investigation.

The pattern of affinities of paludrine for the cholinesterases is similar to that of the substances examined in the preceding paper. Like cocaine, trasentin 6H, and lachesine, paludrine has little affinity for true cholinesterase; pseudocholinesterase is more strongly inhibited and there is a strong inhibition of the benzoylcholinesterase of guinea-pig's liver, whereas the benzoylcholinesterase of the ox kidney is not inhibited. So far as true and pseudo-cholinesterase are concerned, it has been shown that many substances have a greater affinity for the latter enzyme, e.g., di*iso*propyl fluorophosphonate (Hawkins and Mendel, 1947), quinine (Nachmansohn and Schneemann, 1945), and the aromatic amino alcohols of the type $Ar\text{-}CHOH.CH_2NR_2$ recently studied by Wright (1946). It is

interesting that the enzyme specifically connected with the metabolism of acetylcholine in nerve and muscle should be much less readily inhibited.

The authors are grateful to Dr. F. L. Rose for the samples of paludrine and its N_5-methyl homologue used in this investigation.

Two of us (H. B. and I. W.) are indebted to the Medical Research Council for personal grants held while this investigation was carried out.

SUMMARY

1. Paludrine has little affinity for true cholinesterase.

2. It inhibits pseudo-cholinesterase more strongly.

3. It is an inhibitor of the benzoylcholinesterase of guinea-pig's liver, but not of the benzoylcholinesterase of ox kidney.

4. Paludrine, like quinine and mepacrine, has little affinity for the tributyrinesterase of cat's liver, but does inhibit the tributyrinesterase of human serum.

5. The N_5-methyl homologue of paludrine has a much lower affinity for the benzoylcholinesterase of guinea-pig's liver than paludrine itself.

6. The guinea-pig's liver does not contain pseudo-cholinesterase.

REFERENCES

Blaschko, H., Chou, T. C., and Wajda, I. (1947). *Brit. J. Pharmacol.*, **2**, 108.
Curd, F. H. S., and Rose, F. L. (1946). *J. chem. Soc.*, 729.
Fulton, J. D. (1936). *Ann. trop. Med. Parasit.*, **30**, 491.
Glick, D., and Glaubach, S. (1941). *J. gen. Physiol.*, **25**, 197.
Gunter, J. M. (1946). *Nature, Lond.*, **157**, 369.
Hawkins, R. D., and Mendel, B. (1947). *Brit. J. Pharmacol.* (in press).
Mendel, B., Mundell, D. B., and Rudney, H. (1943). *Biochem. J.*, **37**, 473.
Nachmansohn, D., and Schneemann, H. (1945). *J. biol. Chem.*, **159**, 239.
Rona, P., and Pavlovic, R. (1922). *Biochem. Z.*, **130**, 225.
Rona, P., and Reinicke, D. (1921). *Biochem. Z.*, **118**, 213.
Sawyer, C. H. (1945). *Science*, **101**, 385.
Waelsch, H., and Nachmansohn, D. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 336.
Wright, C. I. (1946). *J. Pharmacol.*, **87**, 100.

Brit. J. Pharmacol. (1947), **2**, 121.

OXIDATION OF ADRENALINE IN ALKALINE SOLUTION

BY

G. B. WEST

From the Pharmacological Laboratory, University of Edinburgh

(Received January 8, 1947)

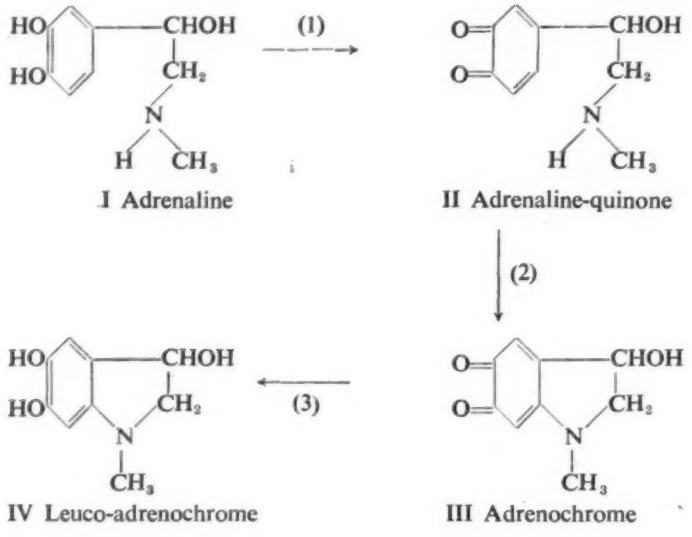
When adrenaline in simple alkaline solutions is exposed to oxygen, the loss of physiological activity is accompanied by various other known phenomena:

1. The solution becomes pink and then red owing to the formation of adrenochrome, which has been isolated in crystalline form and has formula III (Green and Richter, 1937; Richter and Blaschko, 1937). Eventually, this indole quinone is destroyed and the red colour disappears.

2. A green fluorescence develops and then disappears (for references see Jörgensen, 1945). Attention was drawn to the advantages of this fluorescence for the detection of adrenaline by Gaddum and Schild (1934), who found that it provided a sensitive physical test, by which a concentration of 10^{-8} adrenaline could be easily detected. They also found that the reaction was specific at this concentration, many substances chemically related to adrenaline giving no fluorescence, whilst others gave fainter green fluorescences in more concentrated solutions.

3. The solution acquires an increased power of reducing arsenomolybdic acid (Shaw, 1938). This fact has also been made the basis of a specific and sensitive test for adrenaline. If the reducing power of a tissue extract be increased many times by treatment with alkali, the observation can be taken as evidence that the extract contains adrenaline or some other phenol with the same side chain. Shaw stated that, if the treatment with alkali was prolonged, the colour decreased and the more active reducing agent was itself destroyed.

It has been suggested (Utevsky, 1944) that adrenaline undergoes the following chemical changes in these conditions:



I Adrenaline

II Adrenaline-quinone

III Adrenochrome

IV Leuco-adrenochrome

Substance IV eventually undergoes further changes of unknown nature. Substances II and IV have not been isolated, but the theory is supported by a certain amount of indirect evidence.

The object of the experiments described here was to determine the relations which exist between the formation of adrenochrome, the appearance and disappearance of fluorescence, the changes in reducing power and the disappearance of biological activity ; in addition, experiments were carred out in order to obtain evidence about the substances responsible for these different effects and to devise an improved method, based on the fluorescence reaction, of detecting adrenaline in low concentrations.

The fluorescence reaction and chemical properties of the oxidation products of adrenaline

Gaddum and Schild showed that oxidation is the cause of the fluorescence of alkali-treated adrenaline, since none was produced if all oxygen was removed from the solution. Utevsky assumed that oxidation is the cause of the weakening as well, since intense oxidation destroys the fluorescent product. By preparing solutions containing large proportions of each of the reaction products, he was able to study some of their properties. He found that the duration of fluorescence when alkali was added to the orthoquinone (II) or to adrenochrome was shorter than when it was added to adrenaline. Formaldehyde inhibited oxidation of adrenaline to adrenaline-quinone before the indole ring had been

formed. On the other hand, he found that strong fluorescence was produced if adrenochrome and formaldehyde together were treated with alkali, suggesting that the fluorescent material was related to adrenochrome. The conclusion reached by Utevsky was that the material giving the fluorescence was leuco-adrenochrome (IV).

In a limited study, the above reactions have been repeated and confirmed, and so far we have found no evidence to disprove Utevsky's conclusions. The fluorescent material is yellow in solution, gives a positive ferric chloride reaction for catechols, a positive *p*-dimethylaminobenzaldehyde test for indoles, and is adsorbed at *p*H 8.5 by aluminium hydroxide. Efforts to isolate the fluorescent material in the dry state have so far failed, and therefore some of these reactions may be due to impurities. The life of the fluorescent material can be lengthened by the addition of ascorbic acid to a solution with maximal intensity, by adjusting the *p*H of the solution to 6 after maximal production, or by adsorption on alumina at *p*H 8.5.

Quantitative estimation of adrenaline

Much work has been done in adapting the fluorescence reaction for quantitative work. The findings described here were obtained before Jörgensen's work was published, but the conclusions are in good agreement with his.

Gaddum and Schild recommended the use of 5*N*.NaOH for obtaining the fluorescence, but the interval of maximal intensity was then very short. The reading was taken after 20 seconds' contact, and after about one minute no fluorescence remained. The use of more

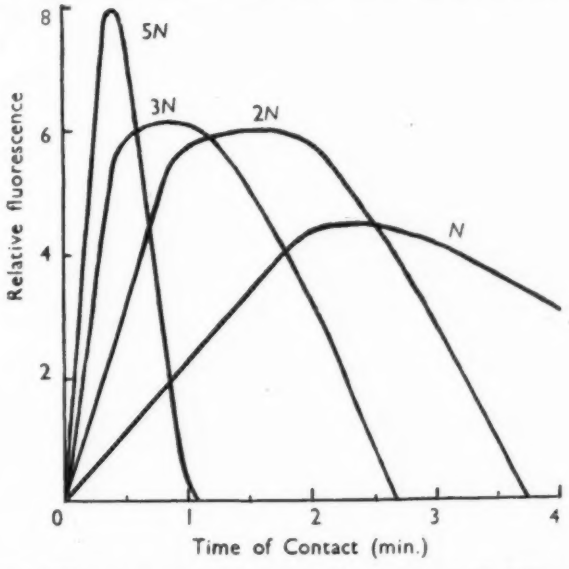


FIG. 1.—The effect of different concentrations (*N* to 5*N*) of NaOH on the intensity and duration of fluorescence of adrenaline solutions. Ordinates: relative fluorescence as measured on the fluorimeter. Abscissae: time of contact between adrenaline (10^{-7}) and alkali (1/10 vol.).

dilute alkali was investigated, therefore, in order to prolong this maximum and enable comparisons to be carried out more accurately. The results of this work, shown in Fig. 1, indicated that a satisfactory strength was 2*N*.NaOH, with which maximal fluorescence was obtained in 1 min. and lasted for 1 min.; only a slight loss of sensitivity resulted. Comparisons were made by two methods: in the first (hereinafter referred to as West's method), the adrenaline fluorescence was compared in Rimington's (1943) fluorimeter against standard eosin solutions, whilst the second was a modification of Jörgensen's method, employing one concentrated standard eosin solution. In the fluorimeter, blank readings for water and alkali only were high, and relative fluorescence readings were obtained by subtracting the scale readings from the blank ones. A linear relationship existed between these readings and the concentration of adrenaline in the solution, for each standard eosin solution used (Fig. 2). Fluorescence was produced by treating 3 ml. of the adrenaline solution with 0.3 ml. of 2*N*.NaOH. The two standard eosin solutions had fluorescent intensities corre-

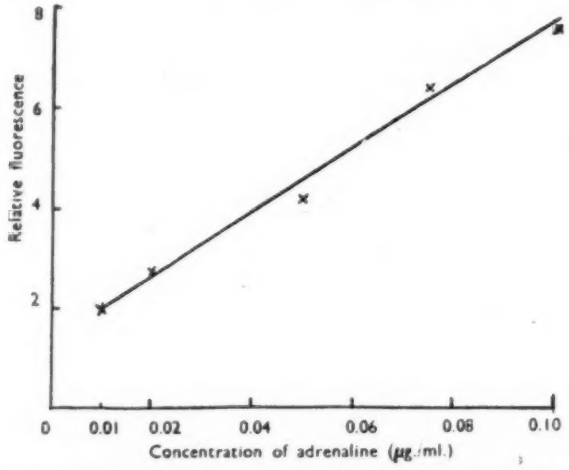


FIG. 2.—Calibration curve for fluorescence of alkali-treated adrenaline, using standard eosin solution "A."

sponding to alkali-treated 10^{-7} and 10^{-8} adrenaline respectively. Monax glass test-tubes have been used throughout this work, as they do not fluoresce in ultra-violet light.

In the second method, the fluorescence was compared with a similar volume of distilled water to which eosin solution (0.1 mg./100 ml.) was added from a burette. Another standard curve was obtained, the volume of eosin solution added being linearly related to the adrenaline content of the solution (Fig. 3). The method was improved by the use of

TABLE I

ESTIMATIONS OF "UNKNOWN" SOLUTIONS OF ADRENALINE BY THE TWO FLUORIMETRIC METHODS

Readings taken after contact with alkali for 1 min. Each experimental figure represents the average of six estimations.

	Adrenaline in μg./ml.									
Solution taken	1.000	0.800	0.500	0.300	0.200	0.100	0.070	0.050	0.030	0.010
Found, West's method	1.010	0.810	0.476	0.303	0.220	0.100	0.074	0.048	0.030	0.014
„ Jörgensen's method	1.020	0.830	0.542	0.300	0.220	0.098	0.075	0.055	0.033	0.012

less alkali (0.2 ml. of 2*N*.NaOH) and less water (2.0 ml. of water), and by carrying out comparisons in the fluorimeter. Solutions of adrenaline prepared by an independent worker were estimated by both methods and good agreement found. The results are recorded in Table I, each figure representing the average of six determinations. The standard error of the test, calculated by the method of Gaddum (1938) using the true values of the " unknown " solutions, was 2.11 per cent with West's method and 2.15 per cent with Jörgensen's method. The limits of error (P = 0.99), therefore, are 100 ± 5.44 and 100 ± 5.53 per cent respectively.

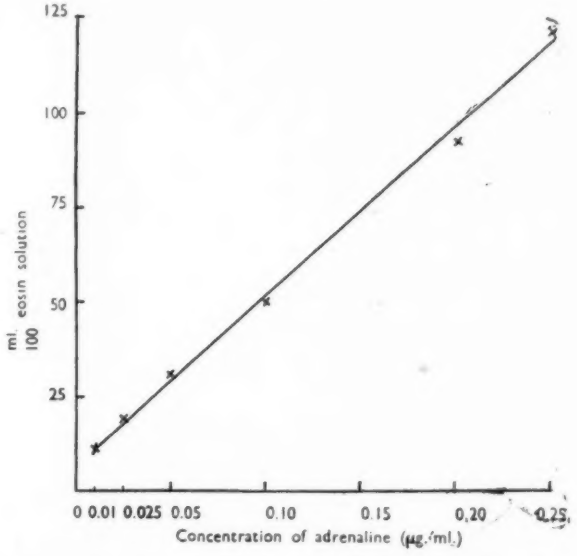


FIG. 3.—Calibration curve for fluorescence of alkali-treated adrenaline, using Jörgensen's eosin solution.

The effect of alkali on strong solutions of adrenaline

In order to measure the biological activity at different stages of the reaction, a more concentrated solution of adrenaline than previously used was treated with a weaker alkali, such as 1.5*N*.Na_2CO_3 (as suggested by Shaw, 1941). The activity was determined using isolated rabbit gut, the perfused frog heart and perfused frog blood vessels, and results are shown in Fig. 4. A loss of 50 per cent was found after 15 min. contact. Chemical estimations by Shaw's (1938) method with and without sodium sulphite and fluorimetric estimations were also carried out.

All the results are reduced to one scale and plotted on one graph (Fig. 5). This graph suggests that the increase in reducing power is not due to the formation of the fluorescent substance (as Utevsky suggested), since maximal fluorescence only appeared after 20 min., and lasted only 5 min. There was a sudden increase in reducing power within 2 min. and this increase (usually five-fold) was maintained for at least 30 min. When the sulphite was omitted from Shaw's test, the values followed the physiological activities very closely, but less than 10^{-6} adrenaline could not be detected by this procedure. There appears to be little or no connexion between loss of physiological activity (measuring rate of

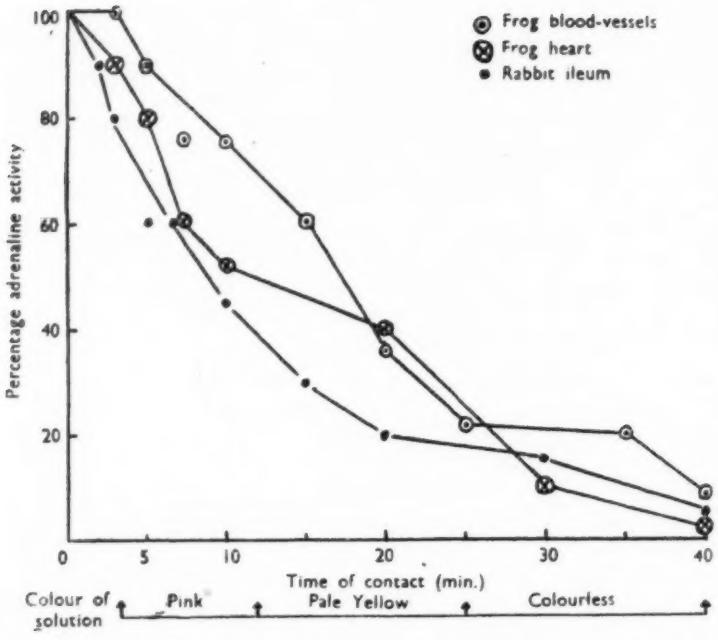


FIG. 4.—The physiological activity of adrenaline (10^{-6}) treated with 1/10 vol. 1.5*N*.Na_2CO_3 for given times. The three methods of assay show fair agreement.

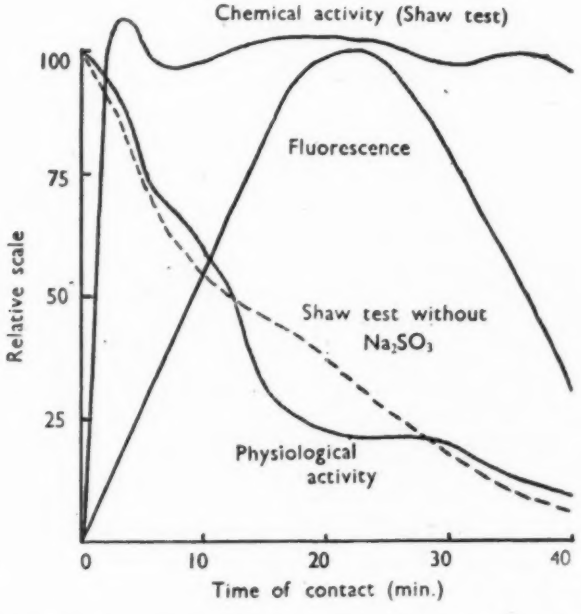


FIG. 5.—The chemical and physiological activities and the fluorescence of adrenaline solution (10^{-5}), when treated with 1.5*N*.Na_2CO_3 for given times.

destruction of adrenaline) and increase in reducing power or production of fluorescence.

Sensitization of tissues to adrenaline by alkali-treated adrenaline

During tests of the physiological activity of alkali-treated adrenaline solutions on the perfused frog blood vessels, an interesting phenomenon was observed. In this preparation, the two anterior venae cavae and the right aorta were ligatured, and Clark's Ringer solution from a Mariotte bottle was perfused via a special arterial cannula into the left aorta and collected from the posterior vena cava into a drop timer (Gaddum and Kwiatkowski, 1938). After about 4 hours' perfusion, during which time the preparation became much more sensitive to doses of adrenaline, consistent responses to 0.01–0.05 μg. were obtained. The presence of oedema was not detrimental to the responses, which were graded according to the dosage (Fig. 6). The perfused blood vessels of female Winter frogs proved the most suitable test objects; moreover, they were relatively insensitive to histamine, acetylcholine and atropine in moderate doses.

A short-lived sensitization of the preparation to a subsequent dose of adrenaline was shown by the same dose of adrenaline previously treated with weak alkali ($1.5N.Na_2CO_3$) for 20 to 25 min. (Fig. 7). No such sensitization was detected in similar experiments when the adrenaline was treated with alkali for 15 min. or less, or for more than 30 min. The time relations suggest that this sensitization is due to the fluorescent substance (Fig. 5).

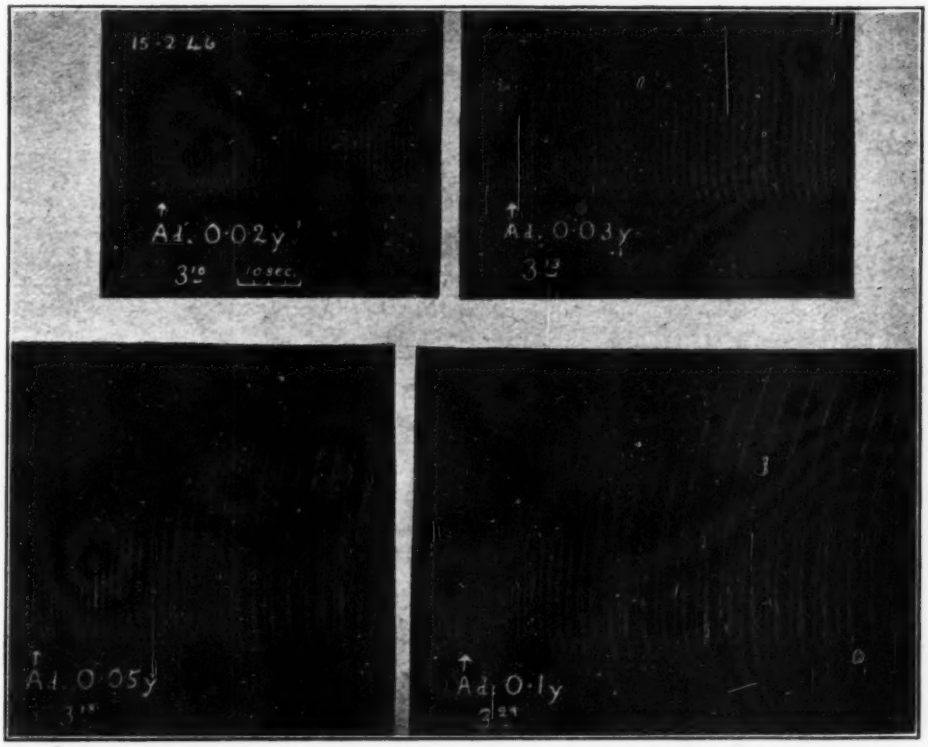


FIG. 6.—Record of outflow of perfused frog blood vessels; the dose-response tracing for adrenaline (Ad.).

because it was only short-lived. That it was not due to the volume of saline injected, the alkali used, or the adrenaline remaining active was shown by injecting these solutions alone just before the adrenaline dose. Jang (1940) has already reported a short-lived sensitization of adrenaline responses by tyramine, sympatol and adrenalone, but a prolonged sensitization

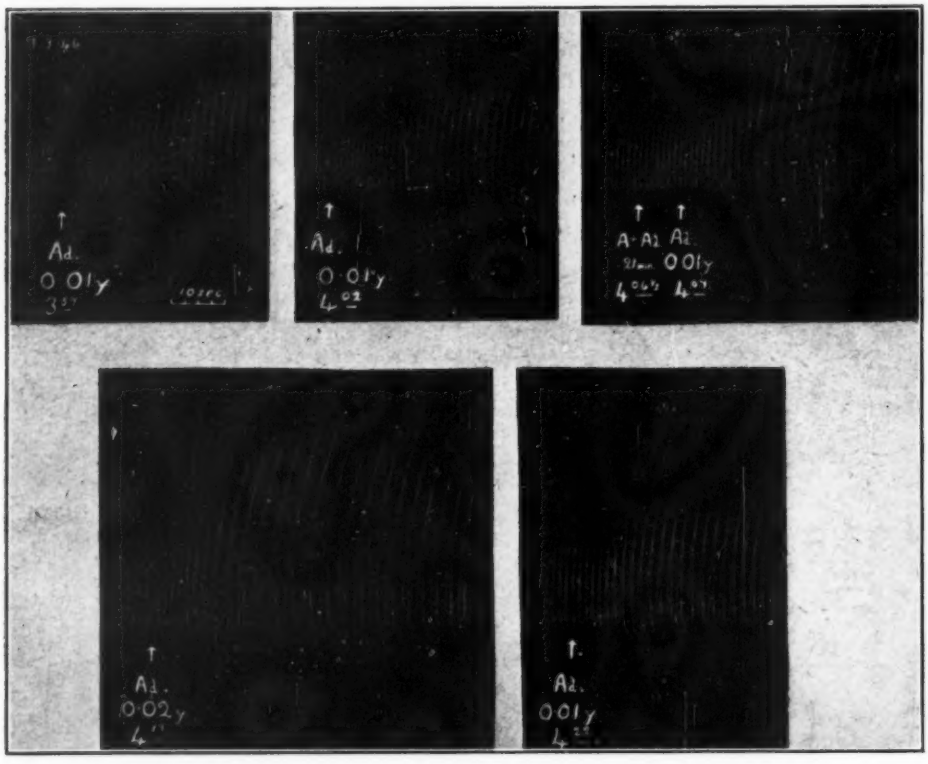


FIG. 7.—The effect of adrenaline, treated with 1.5*N*.Na_2CO_3 for 21 min., and given 1/2 min. before the standard dose of adrenaline (Ad. 0.01 μg.), on the outflow of the perfused frog blood vessels. Enhancement shown was not quite equal to that due to doubling the standard dose.

by phenyl*iso*propylamines such as benzedrine and ephedrine, which are immune to amine oxidase. In the case of the alkali-treated adrenaline, there was some sensitization of the adrenaline response on the spinal cat, rabbit gut, and the Straub frog heart, but none on the perfused frog heart. On the cat's nictitating membrane depression of the adrenaline response sometimes occurred.

DISCUSSION

It is well known that adrenaline is unstable when dilutions are made in Tyrode or Locke solutions. The pink and red colours appear and with them is some fluorescence. Red oxidation products have formed the basis of many chemical methods of estimating adrenaline in solution, the common oxidizing

agents used being ferric chloride, potassium permanganate, persulphate, iodate or dichromate, and iodine. It must not be forgotten, however, that oxidation may occur in the side chain (amine oxidase of Blaschko, Richter, and Schlossmann, 1937).

The fluorescence of adrenaline in alkaline solution has been investigated and most of Utevsky's findings confirmed. That the effect of alkali on adrenaline should be oxidation followed by reduction is an unusual observation, but one explanation might be that a little adrenochrome is first formed and this is reduced at the same time as further adrenaline molecules are oxidized. The process may go on until all the initial adrenaline is oxidized. On the other hand, it is possible that leuco-adrenochrome is formed from adrenaline-quinone and is then oxidized to adrenochrome, in which case the fluorescent material would be another derivative of adrenochrome, giving both catechol and indol reactions.

It has been suggested that the fluorescent reaction is dependent upon a certain grouping such as $\equiv C-CH_2-N=$ (Jörgensen, 1945), or even the phenylethylamine nucleus. Sympathomimetic amines similar to adrenaline, such as *p*-sympatol, epinine, noradrenaline, and "Dopa," produce the same green fluorescence in much stronger solutions (Gaddum and Schild, 1934), but tyramine, ephedrine, and benzedrine do not. Catechol itself produces traces of fluorescence in alkaline solution, so that the basis of the production is not clear. Nevertheless, the test is a specific one for adrenaline in concentrations of 10^{-8} and above, and, when used in conjunction with chemical and biological assays, it can form a good method for the identification of sympathomimetic amines.

From the results shown here, it is certain that the reducing agent in Shaw's test is not entirely composed of the fluorescent material. The brief preliminary treatment of adrenaline with *N*.NaOH for 2 min. produced fluorescence, but the acid nature of the subsequent reagents soon removed any fluorescence before reduction commenced.

Summary

1. Adrenaline in alkaline solution has been shown to produce the fluorescent material, which is probably leuco-adrenochrome, and the reactions leading to its production have been studied. This fluorescent test of Gaddum and Schild has been made of greater use by decreasing the concentration of alkali to 2*N*.NaOH and measuring the fluorescence in a simple fluorimeter.

2. A modified technique for perfusing frogs is described. This preparation is particularly suitable for the assay of adrenaline in tissue extracts since it is relatively insensitive to histamine, acetylcholine, and other substances in these solutions.

3. Fluorescent solutions obtained from adrenaline sensitized the perfused blood vessels of the frog to a subsequent dose of adrenaline, but had little action on cat preparations and rabbit gut.

I wish to express my indebtedness to Prof. J. H. Gaddum for laboratory facilities and valued suggestions.

This work was done while the author was in receipt of a grant from the Wellcome Foundation, for which he desires to express his thanks.

REFERENCES

Blaschko, H., Richter, D., and Schlossmann, H. (1937). *Biochem. J.*, **31**, 2187.
Gaddum, J. H. (1938). *Quart. J. Pharm.*, **11**, 697.
Gaddum J. H., and Kwiatkowski, H. (1938). *J. Physiol.*, **84**, 87.
Gaddum, J. H., and Schild, H. (1934). *J. Physiol.*, **80**, 9P.
Green, D. E., and Richter, D. (1937). *Biochem. J.*, **31**, 596.
Jang, C. S. (1940). *J. Pharmacol.*, **70**, 347.
Jörgensen, K. S. (1945). *Acta pharmacol.*, **1**, 225.
Richter, D., and Blaschko, H. (1937). *J. chem. Soc.*, 601.
Rimington, C. (1943). *Biochem. J.*, **37**, 137.
Shaw, F. H. (1938). *Biochem. J.*, **32**, 19.
Shaw, F. H. (1941). *Austral. J. exp. Biol. med. Sci.*, **19**, 151.
Utevsky, A. M. (1944). *Advances in Modern Biology*, **18**, 45 (in Russian).

Brit. J. Pharmacol. (1947), **2**, 131.

THE ACTION OF ACETYLCHOLINE, ADRENALINE AND OTHER SUBSTANCES ON THE REFRACTORY PERIOD OF THE RABBIT AURICLE

BY

F. J. DE ELÍO

From the Department of Pharmacology, Oxford

(Received January 17, 1947)

In discussing the action of quinidine, veratrine, and strophanthin on the refractory period of cardiac muscle, Lewis and Drury (1926) pointed out that the effect on the absolute refractory period differed from that on the effective refractory period. The absolute refractory period is the length of time before the muscle will again respond to a stimulus; the effective refractory period is the time before the muscle will again transmit a propagated wave to any distance, and for most purposes it is this effective refractory period which is important. Thus Drury and Love (1926) found that quinidine actually shortened the absolute refractory period of the tortoise heart, but lengthened the effective refractory period; it is, of course, this action on the effective refractory period which makes quinidine of value to restore the normal rhythm in auricular fibrillation.

A method of measuring the effective refractory period in the auricular muscle of the rabbit heart has recently been described by Dawes (1946). Using this method to test different substances for their power to act as substitutes for quinidine and to prolong the refractory period of the auricular muscle, Dawes found that substances with several different pharmacological actions were effective. Thus the local anaesthetics amethocaine, cocaine, and procaine prolonged the refractory period; similarly, substances called spasmolytics, such as syntropan, trasentin, pethidine, and papaverine, also prolonged the refractory period. Dawes pointed out that procaine was not only a local anaesthetic, but had a spasmolytic action, shown by its power to reduce the stimulant action of acetylcholine on the rabbit intestine, and further that procaine diminished the action of acetylcholine in inhibiting the amplitude of contraction of the rabbit auricles beating spontaneously. Dawes states that it is remarkable that quinine, quinidine, and procaine antagonize the action of acetylcholine on many different types of tissue. He quotes Harvey (1939a, b), who showed that quinine and procaine reduced the action of acetylcholine on denervated skeletal muscle; he quotes Macgregor (1939), who showed that procaine reduced the pressor response

to acetylcholine in the atropinized cat, and also Stavraky (1932), who showed that quinine reduced the secretory action of acetylcholine on the salivary gland. It appears that many of the substances which prolong the refractory period have the general property of antagonizing the action of acetylcholine.

The question therefore arose what the action of acetylcholine itself on the refractory period of the auricular muscle might be, and the work described here concerns this action. Observations have recently been made by Wedd and Blair (1945) on the action of acetylcholine and adrenaline on the turtle ventricle. They studied the changes in the Q–T interval of the electrocardiogram. With concentrations of acetylcholine equal to 1 in 100,000 or higher, they observed slight shortening of the Q–T interval. In an earlier paper, Blair, Wedd and Young (1941) discussed the relation of the Q–T interval to other events in the cardiac cycle, and concluded that the Q–T interval coincided with the absolute refractory period. Wedd and Blair therefore consider that since acetylcholine and also carbaminoylcholine reduce the Q–T interval, they also reduce the absolute refractory period. In the paper by Drury and Love (1926) in which they distinguished between the refractory period as ordinarily measured and the absolute refractory period, it was shown that whereas quinidine lengthened the refractory period as ordinarily measured, it diminished the absolute refractory period. It is therefore clear that no conclusion can be drawn about the effect of a drug upon the effective refractory period from its effect upon the absolute refractory period.

Dawes (1946) has already stated that adrenaline diminished the refractory period when it was tested on the isolated rabbit auricle. He did not, however, publish any figures relating the dose to the effect.

Experimental Results

Acetylcholine.—The preparation of the rabbit auricle was the same as described by Dawes (1946).

To test the effect of acetylcholine, 12 observations have been made in 4 preparations. As an example of the effect, before the addition of acetylcholine the auricle was able to follow stimulation at the rate of 260 per min., but not at the rate of 274 per min.; each rate was applied twice with the same result. After 10 μg. acetylcholine had been added, the preparation followed stimulation first at 274 per min., then at 292 per min., but failed to follow at 314 per min.; it then followed again at the rate 292 per min. Thus a concentration of 1 in 10 million shortened the refractory period by 12 per cent. Larger doses produced a greater effect, and the addition of 50 μg. enabled the auricle to follow a rate 40 per cent greater than the previous maximal rate. The different observations are given in Table I. In all the diminution of the effective refractory period is clearly seen. The relation between the percentage increase in the maximal rate at which the auricles would follow the imposed rhythm was found to be in linear

TABLE I

EFFECT OF ACETYLCHOLINE IN SHORTENING THE REFRACTORY PERIOD

Experiment No.	Dose μg.	Rate per min. at which auricle followed		Increase %
		before acetylcholine	in presence of acetylcholine	
1	2.5	292	338	15.7
	5.0	292	350	19.8
	10.0	292	363	24.0
2	50.0	284	400	40.9
	50.0	284	362	27.4
3	5.0	314	338	7.5
	10.0	314	362	15.2
	20.0	314	362	15.2
	40.0	314	388	24.0
	40.0	314	388	24.0
	80.0	314	414	32.0
4	10.0	260	292	12.3

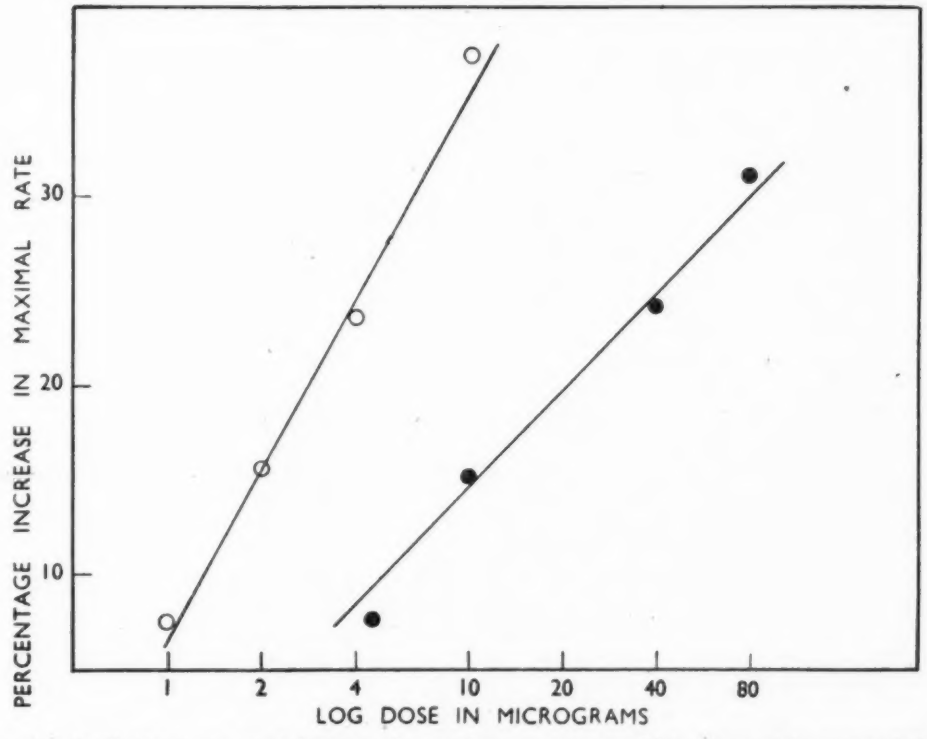


FIG. 1.—Ordinates; per cent increase in maximum rate at which the auricle responded to electrical stimuli. Abscissae: doses (bath of 100 ml.) on a logarithmic scale. The black circles show the effect of acetylcholine; the white circles show the effect of carbaminoylcholine.

proportion to the logarithm of the concentration of acetylcholine in one experiment. This is shown in Fig. 1. The doses of acetylcholine used diminished the amplitude and the frequency of the spontaneous contractions of the auricles, though even the largest dose, which corresponded to a concentration of 1 in 1,250,000, did not cause complete arrest.

Carbaminoylcholine.—Wedd and Blair (1945) found that carbaminoylcholine diminished the Q–T interval of the turtle ventricle, and I have found that, like

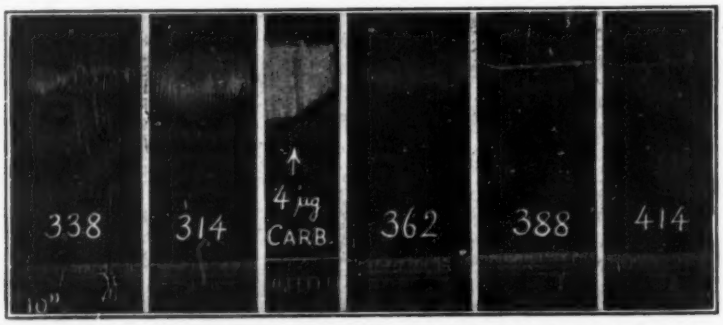


FIG. 2.—Isolated rabbit auricles driven electrically. On the left the auricles fail to follow 338 stimuli per min., but follow 314 per min. regularly. At the arrow 4 µg. carbachol was added to the bath. This diminished the amplitude. The auricles then followed 362 and 388 stimuli per min., though at this high rate the amplitude was very small. At 414 per min. the auricles did not follow the stimuli.

acetylcholine, carbaminoylcholine increases the maximum rate at which the auricles will follow an applied stimulus. The spontaneous contractions of the auricles were arrested, but the responses to the electrical stimulation were more

TABLE II

EFFECT OF CARBAMINOYLCHOLINE IN SHORTENING THE REFRACTORY PERIOD

Experiment No.	Dose µg.	Rate per min. at which auricle followed		Increase %
		before carbaminoylcholine	in presence of carbaminoylcholine	
2	50	292	430	47.2
5	25	252	304	20.6
	50	244	304	24.5
6	1	314	338	7.5
	2	314	362	15.5
	4	314	388	22.5
	8	314	430	37.7
	10	314	430	37.7
7	1	292	338	15.7
	2	292	338	15.7
	4	292	362	23.9
	8	292	388	32.7
	16	292	412	41.0

vigorous than usual. A concentration of 1 in 100 millions increased the maximal rate by 5.5 per cent, and concentrations in the neighbourhood of 1 in 10 millions increased the maximal rate by about 40 per cent. The observations are given in Table II, and the relation between the logarithm of the dose and the effect in one experiment is shown in Fig. 1. An illustration of the effect on the auricles is given in Fig. 2.

Adrenaline.—To determine the effect of adrenaline on the maximal rate of stimulation was a more difficult matter. As already mentioned, Dawes found that adrenaline enabled the auricle to follow a higher rate than before and therefore shortened the refractory period. In most experiments I have confirmed this observation, but in others adrenaline had the opposite effect, at least at certain points (see Table III). Out of 28 observations, adrenaline enabled the

TABLE III

IMMEDIATE EFFECT OF ADRENALINE ON REFRACTORY PERIOD

Experiment No.	Dose μg.	Rate at which auricle followed			Immediate change %
		before adrenaline	in presence of adrenaline	after one washing	
8	10	274	292	260	6.2
	20	274	314	284	14.6
	40	274	378	292	19.0
	80	292	378	—	29.4
9	10	362	338	292	–6.6
	10	292	274	274	–6.1
	20	274	292	274	6.2
	40	274	314	314	14 6
	80	274	292	274	6.2
	160	274	314	274	14.6
	300	274	292	274	6.2
	600	274	274	—	0
10	20	260	274	260	5.3
	40	260	274	260	5.3
11	10	314	338	274	7.6
	10	314	338	274	7.6
	20	314	362	338	15.3
	20	314	338	292	7.6
	40	274	314	292	14.6
	80	292	314	292	7.5
12	10	274	292	244	6.2
	10	260	292	260	12.3
	20	274	338	260	23.3
	40	274	314	234	14.6
13	10	274	274	260	0
	20	274	274	244	0
	40	274	292	274	6.5
	40	274	292	260	6.5

auricle to follow at a higher rate in 23, but in the remaining 5 observations the rate was unaffected or reduced. As examples of reduction, the addition of 10 μg. adrenaline to the bath of 100 ml. reduced the rate from 362 to 338 per min. and, in a second trial, from 292 to 274 per min. The changes in the maximum rate

TABLE IV

THE NUMBER OF TIMES A GIVEN CHANGE WAS OBSERVED

Dose of adrenaline μg. in 100 ml.	Maximum rate			
	increased more than 10%	increased less than 10%	not changed	decreased
10	1	4	1	2
20	3	3	1	
40	4	3		
80	1	2		

caused by adrenaline are shown in Table IV, in which it is seen that as the concentration of adrenaline was increased more observations were made in which the maximum rate was increased. Thus when 10 μg. was added to the bath, an

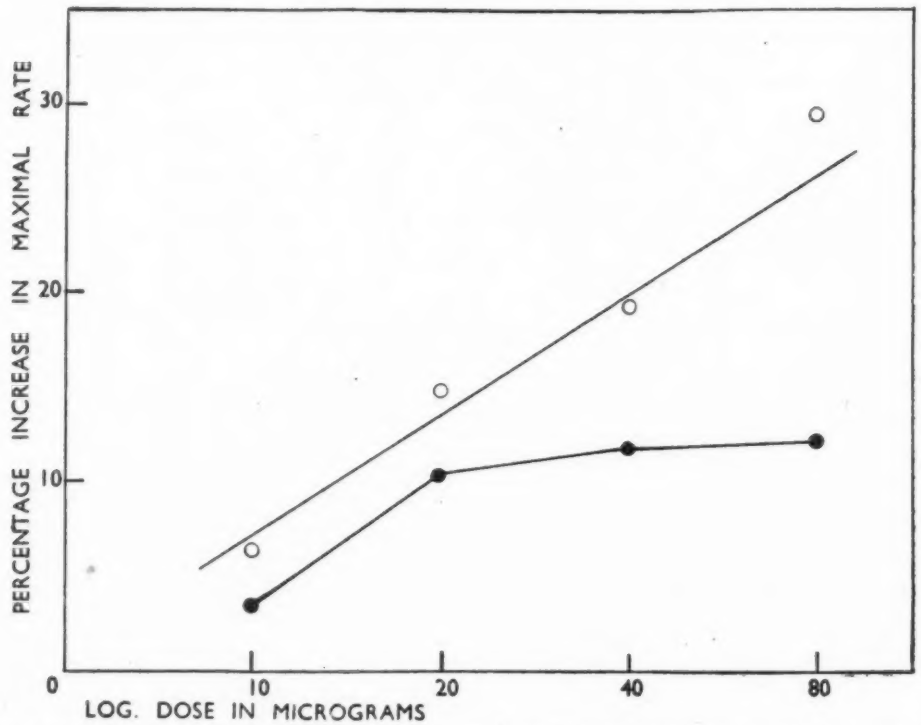


FIG. 3.—Ordinates and abscissae as in Fig. 1. The white circles show the results obtained with different concentrations of adrenaline in one experiment. The black circles are the mean results of all the observations made with adrenaline.

increase greater than 10 per cent was observed in 1 out of 8 trials, whereas when 40 μg. was added such an increase was observed in 4 out of 7 trials. The mean of all observations is shown in Fig. 3, in which the results of one exceptional experiment are also shown where the percentage increase in maximum rate was in linear relation to the logarithm of the dose. In some experiments the higher doses of adrenaline produced a smaller increase in maximal rate than lower doses.

A further curious observation was made that when adrenaline was added to the bath and then washed out, the maximum rate fell below the initial rate; thus the refractory period was first shortened by adding adrenaline, and then lengthened by washing it out. After further washing the rate returned by stages

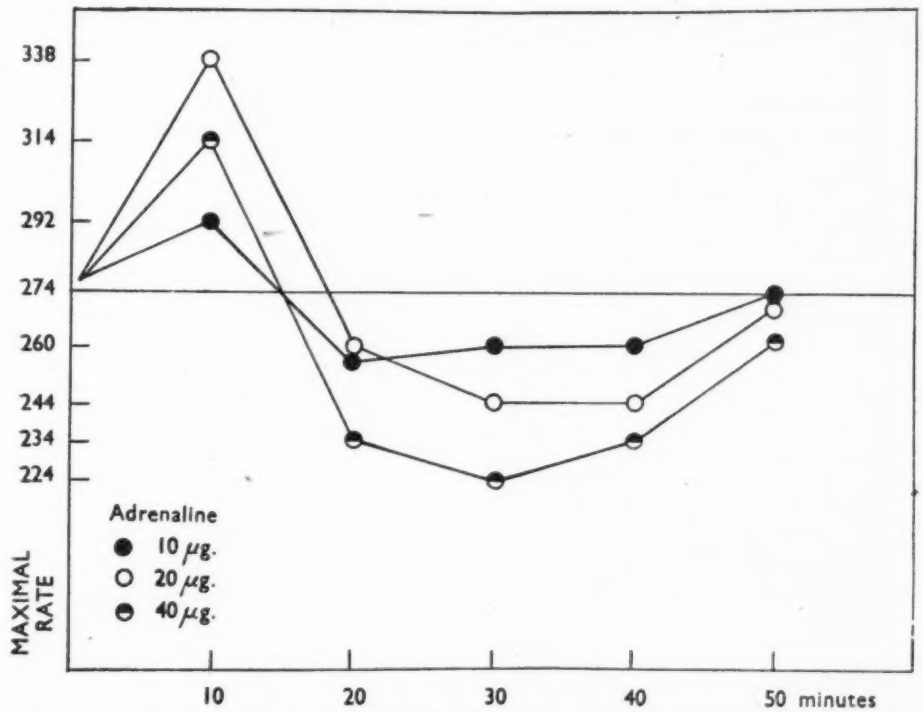


FIG. 4.—Ordinates: maximum rate per min. at which the auricles responded to electrical stimuli. Abscissae: time in minutes. The adrenaline added to the bath was in contact with the auricles only during the first 10 min.; after washing out at 10 min. intervals, the maximum rate fell below the initial rate, and then gradually returned.

to the initial rate. Examples of this effect are given in Fig. 4. This succession of changes caused by adrenaline was observed 9 times in 4 different experiments.

Nicotine.—An action of nicotine (used as acid tartrate) on the isolated rabbit auricle was exerted when large doses were given; these produced an increase in amplitude as shown in Fig. 5; no inhibitory effect was seen. Doses less than 0.8 mg. (added to the bath of 100 ml.) had no effect on the refractory period,

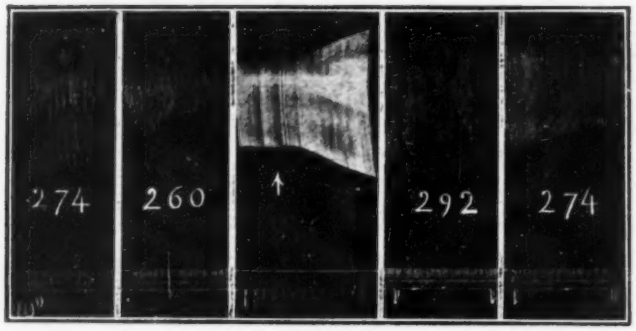


FIG. 5.—Similar to Fig. 2. This record is shown to demonstrate the effect of 1.6 mg. nicotine acid tartrate added at the arrow. Note the increase in amplitude similar to that produced by adrenaline; no inhibition is seen. After the addition of nicotine the auricles followed 274 stimuli per min., whereas before the addition the maximum rate was 260 per min.

but doses equal to this, or greater, usually increased the rate at which the auricles followed the applied stimulus, though the effect was small. In several observations, however, this was the initial effect only, and while the nicotine remained in the bath, the rate fell below the starting rate. Among the results given in Table V, there are examples of this double effect in each experiment. Thus in Exp. 4, the addition of 3.0 mg. caused an initial increase from 274 to 292 per

TABLE V

IMMEDIATE EFFECT OF NICOTINE ON THE REFRACTORY PERIOD

Experiment No.	Dose mg.	Rate at which auricle followed			Change (%)
		before nicotine	in presence of nicotine	after one washing	
4	0.4	274	274	274	0
	0.8	274	260	244	− 5.1
	1.6	260	274	274	+ 5.3
	3.0	274	292		+ 6.5
			224	234	−18.2
	1.6	274	244	260	−10.6
13	0.08	274	274	266	0
	0.2	266	266	266	0
	0.8	266	274		+ 3.0
			238	266	−10.5
	1.6	266	284		+ 6.7
			238	266	−10.5
	3.0	266	304		+14.2
			252		− 5.2
14	0.8	314	338	292	+ 7.6
	1.6	292	326		+11.6
			292	314	− 7.5
	3.0	292	314		+ 7.5
			244	314	−16.4
	1.6	314	338	292	+ 7.6

min., followed by a decrease to 224 per min. before washing out. The two phases of the nicotine action appeared to correspond to a stimulant stage and a paralysing stage. Thus, after testing the effect of 3.0 mg., a retrial of the effect of 1.6 mg. produced a fall in the maximal rate, whereas earlier it produced a rise.

Prostigmine.—The effect of prostigmine was tested in doses of 1 mg. and 2 mg. These amounts produced either no effect or slight increases of 5 or 6 per cent in the maximal rate ; they greatly augmented the effects of acetylcholine and of nicotine, as shown in Table VI. Whereas 200 μg. of acetylcholine alone increased the rate by 39 per cent, in the presence of 1.0 mg. prostigmine 10 μg. of acetylcholine increased the maximum rate by 57 per cent.

TABLE VI

EFFECTS IN THE PRESENCE OF PROSTIGMINE

Substance	Dose μg.	Rate at which auricle followed			Change %	
		before drug	in presence of drug	after one washing	(a)	(a) to (b)
Exp. 15						
acetylcholine	200	260	362 (a)	292	39	
prostigmine	1,000	260	274 (a)		5	
acetylcholine	10		430 (b)	260		57
prostigmine	2,000	260	260 (a)		0	
acetylcholine	20		388 (b)	292		49
nicotine	1,600	260	388 (a)	260	49	
acetylcholine	10	260	388 (a)	244	49	
Exp. 16						
prostigmine	1,000	274	292 (a)		6	
acetylcholine	100		388 (b)	314		33
prostigmine	2,000	292	292 (a)		0	
acetylcholine	200		430 (b)	274		47
prostigmine	2,000	274	292 (a)		6	
nicotine	1,500		362 (b)	274		24
prostigmine	2,000	274	292 (a)		6	
nicotine	3,000		362 (b)	274		24
prostigmine	2,000	274	292 (a)		6	
nicotine	1,500		292 (b)	274		0
nicotine	1,500	274	274 (a)		0	

The increases in maximal rate produced by nicotine and recorded in Table V were often small ; in the presence of prostigmine, however, they were very large ; thus 1.6 mg. nicotine added to the auricle when some prostigmine effect remained (after washing out 2 mg. prostigmine), increased the maximum rate by 49 per cent.

An attempt was made to see if large doses of acetylcholine, added in the presence of prostigmine, would depress the maximum rate. No such depression was observed, as shown in the second part of Table VI, though the repetition of large doses of nicotine led to disappearance of their action.

Eserine.—Eserine acted similarly to prostigmine. Doses of 200 μg. were themselves without effect, but in their presence the action of acetylcholine was increased ; thus 1, 2, and 4 μg. acetylcholine produced increases of 14.5, 20, and 39 per cent in the maximal rate. Eserine likewise increased the action of nicotine ; in the presence of 200 μg. eserine, nicotine in a dose of 0.1 mg. increased the maximum rate by 14.5 per cent, but larger doses of 0.2 mg. and 0.4 mg. nicotine tested afterwards had less action, the increase being 6.5 per cent for each dose.

Atropine.—When atropine in a dose of 1 mg. was added to a fresh preparation there was, in each of three experiments, a slight diminution in the maximum rate. After this addition, acetylcholine failed to cause the usual increase. In some experiments it appeared that after atropine, the action of adrenaline disappeared, but this was not always so, and in view of the variation in the response to adrenaline, it was not possible to conclude that atropine affected its action ; nor did atropine modify the action of nicotine.

Discussion

The results obtained in this work are in line with the points brought out by Dawes concerning the substances which resemble quinidine in its action on the electrically driven auricle. Dawes found that local anaesthetics and spasmolytics have a quinidine-like action and that they share the property of antagonizing the action of acetylcholine in stimulating the isolated intestine of the rabbit and in inhibiting the spontaneous contractions of the isolated rabbit auricles. He pointed out that on skeletal muscle also the action of a local anaesthetic like procaine resembles that of quinine. From Dawes's observations the conclusion can therefore be drawn that substances which prolong the refractory period of cardiac muscle are, in general, antagonists of acetylcholine. The inference from this conclusion is that acetylcholine, and substances with a similar action, should shorten the refractory period. The observations described in this paper show that acetylcholine has this action and that carbaminoylcholine has the same action. The effect of acetylcholine is increased in the presence of eserine or prostigmine, and it is abolished in the presence of atropine. The action of adrenaline is similar in direction to that of acetylcholine, but as the dose of adrenaline is increased there is little increase in effect. With acetylcholine there is a linear relation between log dose and the increase in the maximum rate at which auricles can be driven over a considerable range. With adrenaline this is not so ; with increasing doses of adrenaline the increase in maximum rate is much less, and soon reaches a point beyond which no further increase is observed.

Since the diverse substances which have a quinidine-like action on the auricles have the common property of antagonizing the action of acetylcholine, the suggestion arises that the transmission of the impulse in cardiac muscle may be effected by a mechanism in which acetylcholine is a key substance and in which

the rate of transmission is governed by the rate of formation of acetylcholine. Abdon and Hammarskjöld (1944) have demonstrated that rabbit hearts, among other tissues, contain a precursor from which acetylcholine can be liberated, so that such a mechanism is a possibility. The application of acetylcholine to the isolated auricle might then be supposed to facilitate the working of the mechanism by providing acetylcholine ready made. The action of adrenaline could be regarded as an action in which it potentiated the effect of acetylcholine, the extent of the potentiation being limited. Other examples of the potentiation of acetylcholine by adrenaline have been described by Bülbring and Burn (for references, see Burn, 1945). The action of acetylcholine in shortening the refractory period is abolished by atropine, and if acetylcholine is concerned in the transmission of the normal process of excitation, it might be expected that atropine would be a potent substance in prolonging the refractory period. Several examples, however, are known where a normal process mediated by acetylcholine is not appreciably affected by atropine, although atropine abolishes the action of acetylcholine when externally applied. When, however, such a normal process is exaggerated, then atropine removes the exaggeration. Novoa Santos in his textbook of general pathology (1934) describes how auricular fibrillation in a proportion of patients is arrested by atropine ; if it is supposed that in these cases the fibrillation is due to excessive formation of acetylcholine, and that atropine antagonizes the action of this excess, we can then understand how atropine acts. In a similar fashion, we can understand how atropine removes the tremors of paralysis agitans without affecting ordinary voluntary movements. Ordinary movements appear to require the mediation of acetylcholine at the synapses ; spinal reflexes are not, however, modified by atropine. The effect of accumulations of acetylcholine in the spinal cord, whether inhibiting or stimulating, is, however, abolished by atropine (Bülbring and Burn, 1941).

Summary

1. Observations have been made on the isolated auricles of the rabbit driven electrically. Acetylcholine and carbaminoylcholine increase the maximum rate at which the auricles will follow the stimulation applied.

2. The relation between the percentage increase in maximum rate and the logarithm of the dose is linear for both these substances.

3. The effect of acetylcholine is increased by eserine or prostigmine and abolished by atropine.

4. Adrenaline has some effect in increasing the maximum rate of stimulation, but this effect increases only slightly with increase of dose.

5. Nicotine has an initial acetylcholine-like action in large doses, which is followed by the opposite effect, probably owing to partial paralysis.

6. The mechanism of these changes is discussed.

I wish to express my thanks to Prof. J. H. Burn for his guidance and advice throughout this work.

This work was carried out during the tenure of a grant from the Spanish Council for Scientific Research.

References

Abdon, N. O., and Hammarskjöld, S. O. (1944). *Acta Physiol. Scand.*, **8**, 75.
Blair, H. A., Wedd., A. M., and Young, A. C. (1941). *Amer. J. Physiol.*, **132**, 157.
Bülbring, E., and Burn, J. H. (1941). *J. Physiol.*, **100**, 337.
Burn, J. H. (1945). *Physiol. Rev.*, **25**, 377.
Dawes, G. S. (1946). *Brit. J. Pharmacol.*, **1**, 90.
Drury, A. N., and Love., W. S. (1926). *Heart*, **13**, 77.
Harvey, A. M. (1939a). *J. Physiol.*, **95**, 45.
Harvey, A. M. (1939b). *Bull. Johns Hopk. Univ.*, **65**, 223.
Lewis, T., and Drury, A. N. (1926). *Heart*, **13**, 95.
Macgregor, D. F. (1939). *J. Pharmacol.*, **66**, 350, 393.
Novoa Santos (1934). *Manual de Patologia General*. Madrid: Libreria Médica.
Stavraky, G. W. (1932). *J. Pharmacol.*, **67**, 321.
Wedd, A. M., and Blair, H. A. (1945). *Amer. J. Physiol.*, **145**, 147.

Brit. J. Pharmacol. (1947), **2**, 143.

THE INHALATION OF HEXOESTROL

BY

G. L. FOSS AND J. H. GADDUM

From the Experimental Station, Porton, and Edinburgh University

(Received January 20, 1947)

The word gynaecomastia was first used by Galen to describe an enlargement of the male breast (Karsner, 1946). Such a condition may occur after the administration of natural oestrogens (Dunn, 1935). It occurred, with increased pigmentation of the areola and nipple, in a young man treated for acne with injections of oestradiol benzoate (Foss, 1940). Dunn (1940, 1941) reported gynaecomastia after 5 mg. of stilboestrol daily by mouth for about 2–3 months in sexual criminals. Reports have since been made of similar effects after synthetic oestrogen therapy for prostatic carcinoma (Moore, Wattenburg, and Rose, 1945) and for acne (Simon, 1945).

Scarff and Smith (1942) described two cases occurring in a factory where the highly potent synthetic oestrogen stilboestrol was manufactured in quantity, even when stringent measures of protection were adopted. Occupational gynaecomastia then became a recognized entity, and Fitzsimmons (1944) has described the clinical findings in 20 cases, together with the routes of absorption and the preventive measures taken.

In the early months of 1942 we were interested in this industrial hazard, which naturally was causing a little concern among the workers, as in addition to the gynaecomastia there was the real risk of impotence and testicular atrophy, if only of a temporary nature (Dunn, 1940, 1941), and possibly of malignant change occurring in breasts showing "a dangerous degree of epithelial proliferation" (Scarff and Smith, 1942).

After ingestion of natural oestrogens, much of the activity of a given dose is lost by detoxication in the liver, and it was shown by Biskind and Mark (1939) that a pellet of oestrone implanted in the spleen, and thus absorbed via the liver, was considerably less efficient in producing positive vaginal smears in castrated female rats than a pellet of the same size and shape implanted subcutaneously. Stilboestrol and other synthetic oestrogens probably behave somewhat differently and are effective by the mouth.

Workers in factories where synthetic oestrogens are made must therefore be protected against the risk of swallowing small quantities, or of absorbing them by the skin. They are also liable to be exposed to the inhalation of fine dusts contaminated with oestrogens. The work described below was undertaken in

order to assess the effects of such exposure and to compare the effects with those of similar doses by the mouth.

METHODS

Experiments have been made with male human volunteers and with guinea-pigs. With men no special methods were used, but with guinea-pigs the effect was measured.

Male guinea-pigs show well-marked growth of the nipples with development of mammary tissue after a total dose of 0.2 mg. of hexoestrol has been injected subcutaneously every other day for 20 days (Dodds, 1939). In the experiments described here, light-coloured or white guinea-pigs weighing 300–700 gm. were clipped around the nipples, and were then anaesthetized with ether and photographed under constant conditions. The right nipple was held lightly resting on a fixed and rigid millimetre scale, with white divisions on a black background. The source of light was constant, and the same aperture and the same exposure were used each time. These photographs were all enlarged 7 times, and the length of the nipple and the diameter through the centre of its length were measured by comparison with the photographed scale. The volume of the nipple was then calculated on the assumption that it was a cylinder ; the tapering at the tip was roughly balanced by the broadness of the base. The volume of the nipple was measured in this way before treatment and 14 days later, and the percentage increase in volume was calculated.

Hexoestrol was administered in solution in arachis oil, either by stomach tube or by inhalation of the spray of a Collison (1935) inhaler. The spray was directed down a wide glass tube with a large hole in the side of it, partly closed by a rubber diaphragm, through which the guinea-pig's nose was poked. The dose of hexoestrol inhaled by the guinea-pigs was roughly estimated in the following way. The output of the spray was 9.4 litres of air and 120 mg. of oil per minute, so that the concentration of the oil in the cloud breathed by the guinea-pig was about 12.8 mg./litre. It was assumed that the guinea-pigs, which weighed about 500 g., breathed 250 c.c. of air or 3 mg. of oil per minute (Gaddum, 1944).

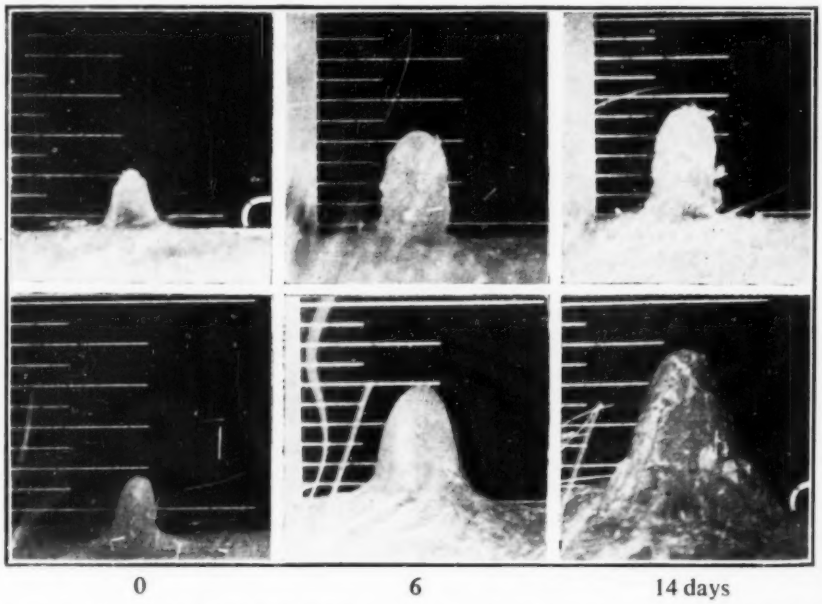


FIG. 1.—Nipples of guinea-pigs before and after a single dose of hexoestrol in arachis oil. Scale, mm. and 0.5 mm.

The dose of hexoestrol was then calculated from the known concentration of the drug in the oil and the duration of the exposure. The assumptions involved make these estimates of the dose unreliable. A small amount of hexoestrol was deposited on the walls of the apparatus and the actual dose inhaled was probably less than the estimate, but it seems likely that it was more than 50 per cent of the estimate.

RESULTS

Guinea-pigs.—In two experiments the effect by the mouth was compared directly with the effect by inhalation. Guinea-pigs which inhaled hexoestrol in the first experiment were not photographed individually before the treatment,

TABLE

GUINEA-PIGS. EFFECTS OF HEXOESTROL ON NIPPLE VOLUME

Weight gm.	Approx. dose μg.	Time of exposure min.	Normal nipples			After 14 days			% inc.	Remarks
			Length mm.	Width mm.	Volume cu.mm.	Length mm.	Width mm.	Volume cu.mm.		
EXPERIMENT 1.										
By mouth using 0.1% and 0.01% solution in Arachis Oil.										
570	50	—	1.7	1.2	1.92	2.9	1.6	5.8	202	
710	100	—	1.8	1.1	1.69	3.4	1.6	6.8	243	
520	250	—	1.5	1.2	1.70	3.3	1.8	8.4	390	
600	500	—	1.7	1.1	1.60	4.0	1.7	9.0	460	
550	750	—	2.2	1.2	2.49	4.8	2.0	15.0	500	
660	1000	—	1.7	1.0	1.34	4.0	1.9	11.2	735	
By inhalation using 1% solution in Arachis Oil.										
355	30	1				3.1	2.4	14	610	
305						—	—	—	—Died 6 days	
305	60	2			1.92	4.3	2.5	21	1000	
400					(Mean	3.9	2.8	24	1150	
390	120	4			of 26)	3.3	2.0	10.4	440	
330						4.0	2.2	15.2	690	
EXPERIMENT 2.										
By mouth using a 0.001% solution in Arachis Oil.										
520	2.5	—	1.8	1.0	1.34	1.8	1.0	1.34	0	
650		—	2.6	1.3	3.4	2.5	1.2	2.8	−18	
550	5	—	2.4	1.2	2.7	2.4	1.1	2.25	−17	
500		—	1.7	1.0	1.33	—	—	—	—Died 5 days	
620	10	—	2.2	1.1	2.1	2.2	1.2	2.5	19	
530		—	2.0	1.1	1.9	2.1	1.1	2.0	5.2	
600	25	—	2.0	1.1	1.9	2.3	1.2	2.6	37	
600		—	2.0	1.1	1.9	2.1	1.2	2.38	25	
700	40	—	2.5	1.5	4.4	—	—	—	—Died 11 days	
520		—	1.9	0.9	1.2	1.9	1.1	1.8	50	
By inhalation using a 0.1% solution in Arachis Oil.										
520	0	0	1.7	1.2	1.9	1.7	1.1	1.6	−16	
530			2.2	1.5	3.9	—	—	—	—Died 16 days	
600	3	1	2.0	1.4	3.1	2.2	1.4	3.38	11	
480			2.2	1.4	3.4	2.5	1.4	3.85	13	
590	6	2	1.8	1.0	1.4	2.8	1.4	4.15	195	
540			1.9	1.3	2.5	2.1	1.6	4.2	68	
500	12	4	2.2	1.2	2.5	4.0	1.8	10.1	300	
420			1.4	0.9	0.88	2.5	1.5	4.4	250	Died 31 days
—	24	8	1.2	0.9	0.75	—	—	—	—Died 16 hr.	
650			1.5	1.3	1.98	2.4	1.6	4.8	143	

and the percentage increase in volume was calculated by comparing the volumes after treatment with the average volume of 26 normal nipples, belonging to guinea-pigs of about the same weight. With this exception the volume of each nipple was estimated before and after treatment. The results of these experiments are shown in the Table and Figures.

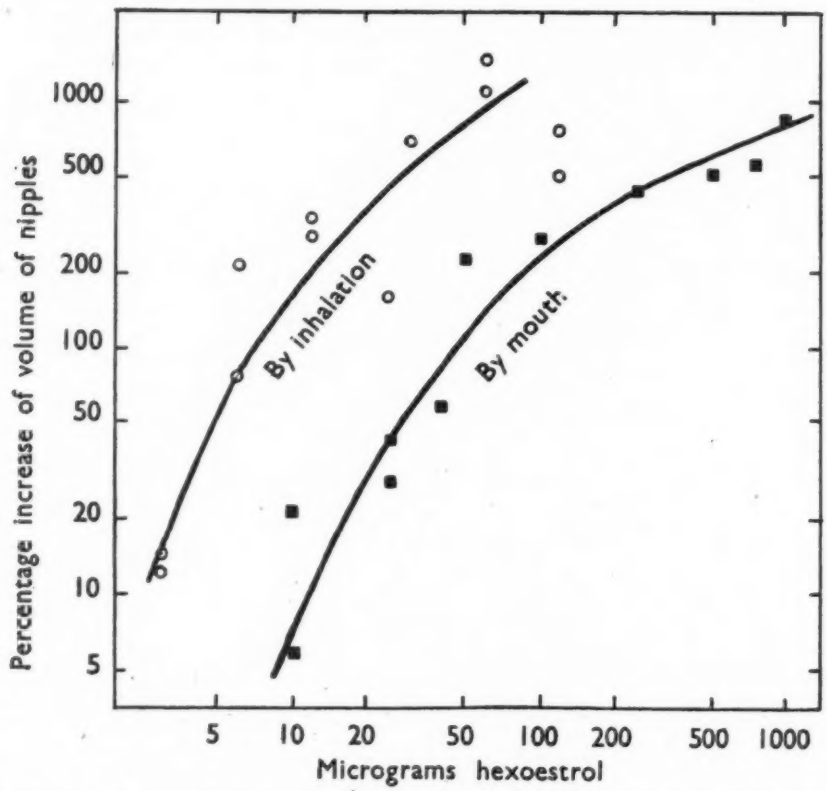


FIG. 2.—Effects of hexoestrol given to guinea-pigs by inhalation (circles) and by the mouth (squares). Logarithmic scales. Horizontally, the estimated dose in micrograms per animal. Vertically, the percentage increase of the volume of the nipples after 14 days.

The deaths recorded in the table were probably not due to hexoestrol. Deaths occurred, among normal guinea-pigs in the stock, rather frequently during this period. The figures are erratic owing to the normal variation between guinea-pigs and the smallness of the numbers used, but they suffice to show approximately the threshold doses for a single dose by the two methods of administration. Hexoestrol in oil was 5–10 times as effective by inhalation as it was by the mouth.

Man.—A series of 7 male observers were given single doses of 15–50 mg. of stilboestrol by mouth without any effect on the nipples or breasts at all. Some of these men, consisting of professors, doctors, and laboratory assistants, noticed

loss of appetite, slight headache, and malaise. One doctor took 55 mg. in 4 days without any effect at all on the nipples. A laboratory assistant swallowed 5 mg. a day for 17 days. He noticed some indigestion and an empty feeling in the epigastrium, and at the end of this time he said that he was conscious of his nipples being rather sensitive and he felt the pressure of his pyjamas on them. On examination the areola were bright pink and there was slight enlargement of the nipples, but there was no thickening of the breast tissue beneath. He then stopped taking tablets, as he felt a little anxious.

Discussion

The experiments on guinea-pigs show that hexoestrol in arachis oil is very effective by inhalation. The effect shown in Fig. 1 was due to exposure for only 2 min. altogether to a concentration of hexoestrol estimated as 128 mg. per cubic metre. The curves shown in Fig. 2 suggest that the dose for a given effect by inhalation is only about 1/5th of the dose for the same effect by oral administration. If the conclusions given above about the error of the estimate of the amount inhaled are true, then the dose by inhalation lies between 1/5th and 1/10th of the dose by oral administration. It is possible that the large effects observed after inhalation were due to the formation of a depot of arachis oil in the lungs from which the hexoestrol was slowly absorbed.

The nipples of man appear to be less sensitive than those of the guinea-pig when hexoestrol is taken by the mouth and when doses are calculated in mg. per kg. Thus a dose of 0.25 mg. or 0.5 mg. per kg., given by the mouth to a male guinea-pig, increased the volume of the nipple five times. The length of the nipple was more than doubled and the general effect was definite, but not maximal. On the other hand, a dose of 50 mg., or nearly 1 mg. per kg., had no observed action on man. The drug is probably more effective when given in repeated small doses, but it is apparent from Dunn's experiment and from those recorded here that 5 mg. per day is only effective if continued for several weeks.

There is no quantitative information available about the effects of inhalation by man, and calculations about this must be based on indirect evidence. A dose of 5 mg. of hexoestrol per day by the mouth is likely to lead to troublesome effects if continued. Judging by the experiments with guinea-pigs, this would be equivalent to between 0.5 and 1 mg. per day by inhalation. If a man inhales 15 litres per minute, he will inhale 7.2 cu.m. in a working day of 8 hours. It would therefore be expected that a concentration of 0.5–1 mg. in 7.2 cu.m., or 0.07–0.14 mg. per cu.m., would be harmful after several weeks if exposure were continuous for 8 hours a day. Fitzsimmons (1944) observed a concentration of 0.2 mg. of stilboestrol in 300 cu.ft., or 0.023 mg. per cu.m., on one occasion in the Glaxo laboratory. Our calculations suggest that such concentrations of hexoestrol would not be immediately harmful, but are near the dangerous limit if maintained for several weeks.

Fitzsimmons found that the vaginal smears of ovariectomized mice became positive when they were kept for 3 days in various parts of the factory where stilboestrol was being prepared, even when careful precautions were taken to prevent the dispersal of the oestrogen in the air. An alternative biological test is now available. If male guinea-pigs were kept in factories where synthetic oestrogens are made, their nipples could be inspected, and if necessary measured, at regular intervals and so used as an index of danger. This would be simpler than the vaginal smear technique, though presumably less sensitive. It is probable, however, that the smear test is too sensitive and becomes positive when there is no danger. There is reason to believe that the guinea-pig's nipple will provide an indicator of oestrogens in the air which is sensitive enough for practical purposes. The guinea-pig is more sensitive than man to hexoestrol as judged by the oral dose per kg. of body weight; he inhales more air, and thus more oestrogen, per kg. of body weight and is thus likely to be relatively even more sensitive to clouds than to oral administration. If a man and a guinea-pig were exposed daily to clouds of oestrogen together, the guinea-pig should therefore be affected first and give adequate warning to the man.

The results also suggest a method for the therapeutic administration of oestrogens. They have been given by ingestion, injection, inunction, and implantation. They might also be given by inhalation, but it would probably be difficult to control the dosage and there would be no obvious compensating advantages in this method of administration.

Summary

1. A solution of hexoestrol in arachis oil was administered to male guinea-pigs and the effect on the nipples was photographed and measured.

2. A given dose had more effect when given by inhalation than when given by the mouth.

3. It is suggested that male guinea-pigs should be kept in places where synthetic oestrogens are made, to give warning of any failure of the measures taken to protect the workers from the effects of the oestrogens.

We are indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish this paper.

References

Biskind, G. R., and Mark, J. (1939). *Johns Hopk. Hosp. Bull.*, **65**, 212.
Collison, W. E. (1935). *Inhalation Therapy Technique.* London: Heinemann.
Dodds, E. C. (1939). *Lancet*, **2**, 312.
Dunn, C. W. (1935). *Amer. J. Obstet. Gynec.*, **30**, 186.
Dunn, C. W. (1940). *J. Amer. med. Ass.*, **115**, 2263.
Dunn, C. W. (1941). *J. clin. Endocrin.*, **1**, 643.
Fitzsimmons, M. P. (1944). *Brit. J. industr. Med.*, **1**, 235.
Foss, G. L. (1940). M.D. Thesis, Cambridge University.
Gaddum, J. H. (1944). *Pharmacology*, 2nd edition. Oxford Univ. Press.
Karsner, H. T. (1946). *Amer. J. Path.*, **22**, 235.
Moore, G. F., Wattenburg, C. A., and Rose, D. K. (1945). *J. Amer. med. Ass.*, **127**, 60.
Scarff, R. W., and Smith, C. P. (1942). *Brit. J. Surg.*, **29**, 393.
Simon, E. (1945). *Union méd. Can.*, **74**, 37.